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PATENT ABSTRACTS OF JAPAN

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(54) MODIFIED BLOOD COAGULATION FACTOR VII

(57)Abstract:
 PROBLEM TO BE SOLVED: To obtain a new (activated) modified blood coagulation factor VII which has a modification at a specific site of an amino acid sequence, has an enhanced enzyme activity, and is useful as a medicine effective for treating a hemophilia inhibitor patient or the like.
 SOLUTION: This is a new (activated) modified blood coagulation factor VII (FVII) which has at least one modification(s) selected from a group comprising cleaving the disulfide bond (159Cys-164Cys) consisting of 159th cysteine (159Cys) and 164th cysteine (164Cys) in blood coagulation factor VII, substituting, adding, or deleting at least a part of the amino acid sequence constituting the loop structure consisting of the amino acid sequence from 233rd threonine (233Thr) to 240th asparagine (240Asp) in FVII, and substituting, adding, or deleting at least a part of the amino acid sequence constituting the intervening sequence consisting from 304th arginine (304Arg) to 329th cysteine (329Cys) in FVII, and has an enhanced enzyme activity.

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CLAIMS

[Claim(s)]

[Claim 1] The alteration field of the blood coagulation factor VII (following, FVII) characterized by including at least one alteration chosen from the following, or activated type blood coagulation factor VII (following, FVIIa).

(a) Cut the disulfide bond (159Cys-164Cys) which consists of the 159th cysteine (159Cys) in FVII, and the 164th cysteine (164Cys).

(b) Replace, add or delete the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ***** (233Thr) in FVII, or its part.

(c) Replace, add or delete the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part.

[Claim 2] The alteration field according to claim 1 characterized by cutting the concerned 159Cys-164Cys by replacing the above-mentioned 159Cys and 164Cys by amino acid residues other than Cys.

[Claim 3] The alteration field according to claim 1 or 2 which consists of an amino acid sequence of array table array number 4 publication.

[Claim 4] The alteration field according to claim 1 characterized by cutting 159Cys-164Cys and forming a disulfide bond (159Cys-299Cys) between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cyses, and replacing the 299th valine (299Val) by Cys.

[Claim 5] The alteration field according to claim 1 or 4 which consists of an amino acid sequence of array table array number 6 publication.

[Claim 6] The alteration field according to claim 1 characterized by being replaced by the amino acid sequence to which the amino acid sequence of 99-loop of FVII corresponds on the structure of other trypsin group serine proteases.

[Claim 7] The alteration field according to claim 1 or 6 other trypsin group serine proteases of whose are Homo-sapiens trypsins.

[Claim 8] The alteration field according to claim 1, 6, or 7 characterized by being replaced by Asp-Arg-Lys-Thr-Leu which has an amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) in the loop structure of corresponding on the structure of a Homo -

sapiens trypsin.

[Claim 9] The claim 1 which consists of an amino acid sequence of array table array number 8 publication, or the alteration field given in either 6-8.

[Claim 10] The alteration field according to claim 1 characterized by being replaced by the amino acid sequence to which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part corresponds on the structure of other trypsin group serine proteases.

[Claim 11] The alteration field according to claim 1 or 10 other trypsin group serine proteases of whose are Homo-sapiens trypsins.

[Claim 12] The alteration field according to claim 1, 10, or 11 with which the amino acid sequence which constitutes the mediation amino acid sequence (170-loop may be called hereafter) of the 329th cysteine (329Cys) from the 310th cysteine (310Cys) in FVII, or its part is replaced, added or deleted.

[Claim 13] The claim 1 characterized by being replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys which has an amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) in the loop structure of corresponding on the structure of a Homo-sapiens trypsin, or the alteration field given in either 10-12.

[Claim 14] The claim 1 which consists of an amino acid sequence of array table array number 10 publication, or the alteration field given in either 10-13.

[Claim 15] The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of corresponding on the structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field according to claim 1 characterized by being replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of corresponding on the structure of a Homo-sapiens trypsin.

[Claim 16] The alteration field according to claim 1 or 15 which consists of an amino acid sequence of array table array number 12 publication.

[Claim 17] The drug constituent which contains the alteration field of a publication as an active principle in either of the claims 1-16.

[Claim 18] A medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent of a claim 17.

DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

[0002]

[A prior art and the technical problem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412- 2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).

[0003] If the enzyme activity of the FVIIa [itself] is very weak and it combines with the tissue factor (TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between bipartite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic

activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique -- respectively -- (1) -- more -- high -- about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [4 / (4) / a thrombus induction of DIC, and] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsume editorial supervisions, educational company issue, 1992). It is considered by the ground [blood coagulation factor] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scanning, 112 Alanine substitution-product *****'s of FVII and the thing which enzyme activity went up in it are only [one], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical problem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

[0012]

[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.

[0013]

[Elements of the Invention] a group similar to a trypsin group -- the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence (drawing 1) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease (drawing 2). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed (drawing 3). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-**) 159Cys from which 159Cys-164Cys combination was cut, and 164Cyses by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-**) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cys, and replacing the 299th ***** (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ***** (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in drawing 3 , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo -sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in drawing 3 -- setting -- the serine protease of others [FVII] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo -sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine (310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo -sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example ***** -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo -sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39)

replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [a gene] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfests a host cell, and is acquired by refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

[0022]

[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawing, it illustrates in the example [****] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [as long as there is no notice especially the following] transgenics etc. is TAKARA SHUZO, Toyobo, and par ***** applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [Molecular Basis] of Thrombosis and cDNA array well-known at Hemostasis (- FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTGCCTCTG) which added Sall site to the array table array number 1 on the basis of written) – and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by Sall and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by Sall and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5, and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by Sall and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared (drawing 6). Moreover, about "pVII-6", the gene obtained in

drawing 5 using primer ** and ** of a publication is used as mold, and it was obtained by performing PCR further using primer ** and **. Moreover, about "pVII-39", the gene obtained using primer ** and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array. [0026] The commercial ***** cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>> above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (***** chromium FVII; Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg [/ml] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [as opposed to SDS-PAGE or commercial FVII for the purified alteration field], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml – as – Tris-BSA – diluting – FVII lack *****, equivalent *****, and 37 degrees C – 3 minutes – warming – equivalent addition of the formation TF (thromboplastin;Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start. The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [protein concentration (it measures by the Bradford method)], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [having freezing activity high two to 6 times] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 μg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys-164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys-299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99 を Trypsin 型へ	3,361	685	4,907	245
VII-31	loop170 を Trypsin 型へ	3,589	877	4,093	205
VII-39	loop99+170 を Trypsin 型へ	8,954	773	11,584	579

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [finishing / refining] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.

[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris-HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible.

Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what **, and showed hydration activity

higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

基質名	構造	水解活性/ $\text{mOD}_{405\text{nm}}/\text{min}$		比 31/W
		VIIa-W	VIIa-31	
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
S-2288	H-D-Ile-Pro-Arg	25.8	304.2	12
S-2366	pyro-Glu-Pro-Arg	11.5	267.8	23
S-2238	H-D-Phe-Pip-Arg	11.3	86.6	8
Chromozym X	D-Nile-Gly-Arg	13.0	48.6	4
S-2302	H-D-Pro-Phe-Arg	7.4	38.3	5
S-2765	Z-D-Arg-Gly-Arg	13.3	20.6	2
Chromozym TRY	CBz-Val-Gly-Arg	5.6	28.2	5
S-2444	pyro-Glu-Gly-Arg	1.5	18.9	13
S-2222	Bz-Ile-Glu-Gly-Arg	5.6	16.4	3
S-2403	pyro-Glu-Phe-Lys	0.3	5.9	19

[0032]

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or VIIa has clearly high cnzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

SEQUENCE-LISTING<110> The-Chemo-Sero-Therapeutic Research-Institute<120> Recombinant-mutants of-blood-coagulation factor VII<160> 12<210> 1<211> 1221<212> DNA<213> blood coagulation factor VII<400> 1GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC-TCC-CTG GAG AGG GAG 48Ala Asn Ala Phe Leu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 15 1015 TGC AAG GAGGAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTC TGG ATTCT TAC AGT GAT GGG GAC 144AspAla Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser SerPro Cys Gln Asn Gly GlySer Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACGGGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAAATA CCT ATT CTAGAA AAA AGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG-AAC-TGG-AGGAAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GCGGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGGCGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Ser Arg Lys Val

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330 335 AAG-GAC-TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC
1056Lys Asp Ser Cys Lys Gly Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr 340 345 350 CGG-GGC
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Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG
GTG TAC ACC AGG GTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg
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Cys HisGlu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro
Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn AlaSerLys Pro Gln Gly Arg IleVal Gly Gly
Lys Val Cys Pro 145 150 155 160 Lys-Gly-Glu-Cys-Pro Trp Gln Val Leu Leu-Leu-Val-Asn-Gly-Ala-
Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser AlaAla180 185190 HisCys
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His Asp Gly Asp Glu GlnSer Arg Arg 210 215 220Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly
Thr Thr Asn 225 230 235 240His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245
250255 HisValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala Phe
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Val Gly Asp Ser 305 310 315320 Pro AsnIleThr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
325 330 335Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350Arg Gly
Thr Trp Tyr Leu ThrGly IleVal Ser Trp Gly Gln Gly Cys 355 360 365AlaThr Val Gly His Phe Gly Val
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His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG
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AAA AGA432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140
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Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro 145 150 155 160 AAA GGG GAG
GCC CCA TGG CAG GTC CTG TTG TTGGTG AAT GGA GCT CAG 528Lys Gly Glu Ala Pro Trp
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AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-
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GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln
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CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAGGAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CACTAC 1056Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350 CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC 1104Arg Gly Thr Trp Tyr-Leu-Thr-Gly-Ile Val Ser Trp Gly Gln-Gly-Cys 355 360 365 GCA-ACC-GTG-GGC-CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val-Ser-GlnTyr Ile 370 375 380 GAGTGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC1200Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400 CTG CGA GCC CCA TTT CCC TAG 1221Leu Arg AlaPro Phe Pro 405 <210> 4 <211> 406<212> PRT<213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which both of the 159th Cysteine and the 164th Cysteine are replaced with Alanine.<400> 4Ala Asn Ala Phe Leu Glu Leu Arg Pro Gly SerLeu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys SerPhe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp GlyAsp 35 40 45Gln Cys Ala Ser Ser Pro CysGln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr 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15 TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTCTGG ATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGGAAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys Glu Th His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT 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Asn Thr Ile Trp Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG-AAC-
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Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG
CCC GAA CGG ACG TTC TCT GAGAGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr
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40 45 Gln Cys AlaSer Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile
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Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala
Ala 180 185 190 His-Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Val-Leu 195 200 205
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Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235240 His AspIleAla Leu Leu Arg Leu His Gln
Pro Val ValLeu Thr Asp 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg
Thr 260265 270Leu Ala Phe Val Arg Phe SerLeu ValSer Gly Trp Gly Gln Leu Leu 275 280
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Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395400 Leu Arg Ala Pro Phe Pro 405
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Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu, and cDNA sequence coding
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AAG-GAG-GAG-CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys
Glu GluGln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG
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Ser Tyr SerAsp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAGAAT GGG GGC TCC

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Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160 AAA GGG GAG TGT CCA TGG CAG
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GTC TCC GCG GCC576Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185
190 CAC TGT TTC GAC AAA ATCAA G-AAC-TGG-AGG-AAC-CTG ATC GCG GTG CTG
624His Cys Phe Asp Lys Ile Lys Asn Trp-Arg-Asn-Leu-Ile Ala Val Leu 195 200 205 GGC-GAG-
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Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTGGCG CAG GTC ATC ATC
CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr
Asp Arg Lys Thr Leu Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTGCACCAG
CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr
Asp 245 250 255CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT
GAGAGGACG 816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270
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GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG912Asp Arg Gly Ala Thr Ala Leu Glu
Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG
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TAC CTG ACG GGC ATCGTC AGC TGG GGC CAG GGC TGC 1104Arg Gly Thr Trp Tyr Leu Thr
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Tyr Ile 370 375 380 GAG-TGG-CTG-CAA-AAG CTC-ATG-CGC-TCA-GAG CCA CGC CCA GGA
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Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro
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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

[0002]

[A prior art and the technical problem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412- 2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).

[0003] If the enzyme activity of the FVIIa [itself] is very weak and it combines with the tissue factor (TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between bipartite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique – respectively – (1) – more – high – about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [4 / (4) / a thrombus induction of DIC, and] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinc structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [blood coagulation factor] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scanning, 112 Alanine substitution-product *****'s of FVII and the thing which enzyme activity went up in it are only [one], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical problem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

[0012]

[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.

[0013]

[Elements of the Invention] a group similar to a trypsin group -- the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence (drawing 1) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease (drawing 2). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate

specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed (drawing 3). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-**) 159Cys from which 159Cys-164Cys combination was cut, and 164Cys by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-**) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cys, and replacing the 299th ***** (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ***** (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in drawing 3, and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in drawing 3 -- setting -- the serine protease of others [FVII] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine (310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo-sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example ***** -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39) replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombinant [a gene] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfects a host cell, and is acquired by

refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

[0022]

[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawing, it illustrates in the example [****] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [as long as there is no notice especially the following] transgenics etc. is TAKARA SHUZO, Toyobo, and par ***** applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [Molecular Basis] of Thrombosis and cDNA array well-known at Hemostasis (-- FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTGCCTCTG) which added Sall site to the array table array number 1 on the basis of written) -- and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by Sall and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by Sall and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5, and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by Sall and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared (drawing 6). Moreover, about "pVII-6", the gene obtained in drawing 5 using primer ** and ** of a publication is used as mold, and it was obtained by performing PCR further using primer ** and **. Moreover, about "pVII-39", the gene obtained using primer ** and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array.

[0026] The commercial ***** cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>>

above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (***** chromium FVII; Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg /ml] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [as opposed to SDS-PAGE or commercial FVII for the purified alteration field], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack *****, equivalent *****, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin;Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [protein concentration (it measures by the Bradford method)], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [having freezing activity high two to 6 times] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 μg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys-164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys-299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99 を Trypsin 型へ	3,361	685	4,907	245
VII-31	loop170 を Trypsin 型へ	3,589	877	4,093	205
VII-39	loop99+170 を Trypsin 型へ	8,954	773	11,584	579

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [finishing / refining] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.

[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris -HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible.

Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what **, and showed hydration activity higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

基質名	構造	水解活性/ $\text{mOD}_{405\text{nm}}/\text{min}$		比 31/W
		VIIa-W	VIIa-31	
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
	H-D-Ile-Pro-Arg	25.8	304.2	12
	pyro-Glu-Pro-Arg	11.5	267.8	23
	H-D-Phe-Pip-Arg	11.3	86.6	8
	D-Nile-Gly-Arg	13.0	48.6	4
Chromozym X	H-D-Pro-Phe-Arg	7.4	38.3	5
	S-2302			
	Z-D-Arg-Gly-Arg	13.3	20.6	2
Chromozym TRY	CBz-Val-Gly-Arg	5.6	28.2	5
	pyro-Glu-Gly-Arg	1.5	18.9	13
	Bz-Ile-Glu-Gly-Arg	5.6	16.4	3
	pyro-Glu-Phe-Lys	0.3	5.9	19

[0032]

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or VIIa has clearly high enzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

SEQUENCE-LISTING<110> The-Chemo -Sero-Therapeutic Research-Institute<120> Recombinant mutants of blood-coagulation factor VII<160> 12<210> 1<211> 1221<212> DNA<213> blood coagulation factor VII<400> 1GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC-TCC-CTG GAG AGG GAG 48Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 15 1015 TGC AAG GAGGAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTC TGG ATTCT TAC AGT GAT GGG GAC 144AspAla Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser SerPro Cys Gln Asn Gly GlySer Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACGGGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTGTCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAAATA CCT ATT CTAGAA AAA AGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG-AAC-TGG-AGGAAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGGCAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGGCAG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAG-GAC-TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC

1056Lys Asp Ser Cys Lys Gly Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr 340 345 350 CGG-GGC
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Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG
GTG TAC ACC AGG GTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg
Val Ser Gln Tyr Ile 370 375 380 GAG TGG CTG CAAAAGCTC ATG CGC TCA GAG CCA CGC
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Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro
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Cys HisGlu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro
Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn AlaSerLys Pro Gln Gly Arg IleVal Gly Gly
Lys Val Cys Pro 145 150 155 160 Lys-Gly-Glu-Cys-Pro Trp Gln Val Leu Leu-Leu-Val-Asn-Gly-Ala-
Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser AlaAla180 185190 HisCys
PheAsp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200205Gly Glu His Asp Leu Ser Glu
His Asp Gly Asp Glu GlnSer Arg Arg 210 215 220Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly
Thr Thr Asn 225 230 235 240His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245
250255 His ValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala Phe
Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 AspArg Gly Ala Thr Ala Leu Glu
Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys
Val Gly Asp Ser 305 310 315320 Pro AsnIleThr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
325 330 335Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350Arg Gly
Thr Trp Tyr Leu ThrGly IleVal Ser Trp Gly Gln Gly Cys 355 360 365AlaThr Val Gly His Phe Gly Val
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AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG
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Thr Lys Leu Phe Trp Ile Ser Tyr SerAsp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC
CAG AAT GGG GGC TCC TGC AAGGAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly
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GGC CGG AAC240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80
TGTGAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys
Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95GGC TGT GAG CAG TAC
TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp
His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG
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AAA AGA432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140
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GCC CCA TGG CAG GTC CTG TTG TTGGTG AAT GGA GCT CAG 528Lys Gly Glu Ala Pro Trp
Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-
AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-
Val-Val-Ser-Ala-Ala 180 185 190 CACTGT-TTC-GAC-AAA ATC-AAG AAC TGG AGGAAC
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195 200 205 GGCGAG CAC GAC CTC ACC GAG CAC GAC GGG GAT GAG CAG AGC
CGGCAG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG
GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln
Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG
CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu
His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG
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Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG
CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT
GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala
Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC
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CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC 1104Arg
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25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp GlyAsp 35 40 45Gln Cys Ala Ser Ser
Pro CysGln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala
Phe Glu GlyArg Asn 65 70 75 80 Cys GluThr HisLys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
85 9095 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys His
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Ala Pro 145 150 155 160 Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170
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Thr Asn 225 230 235 240 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245
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Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 AspArg Gly Ala Thr Ala Leu Glu
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replaced with Cysteine, and cDNA-sequence-coding thereof.<400> 5GCC-AAC-GCG-TTC-CTG-
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GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe
Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTCTGG ATT TCT TAC AGT GAT GGG
GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT
GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser
Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC
TGC CTC CCT GCC TTC GAG GGC CGGAAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala
Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG
AAC GAG AAC GGC 288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90
95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT 336Gly
Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG
GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser
Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA
ATA CCTATT CTA GAA AAAAGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu
Lys Arg 130 135 140 AAT GCC AGC AAACCCCAA GGC CGA ATT GTG GGG GGC AAG GTG
TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160
AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG GTG AAT GGA GCT CAG 528Lys
Gly Glu Ala Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175 TTGTGT GGG GGG

ACC CTG ATC AAC ACC ATC TGG GTG GTC TCCGCG GCC576Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG-AAC-TGG-AGG AAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys-Asn-Trp-Arg-Asn-Leu-Ile-Ala-Val-Leu 195 [200] 205 GGCGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGGCAG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG GCG CAG GTCATC ATC CCC AGC ACG TAC GTCCCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768HisAsp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAGAGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC AGC GCC CTG GAG CTC ATG TGC CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGGAAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAGGAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC 1056Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350 CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGGGGC CAG GGC TGC 1104Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG GTG TAC-ACC-AGG-GTC-TCC CAG TAC ATC 1152Ala-Thr-Val-Gly-His Phe Gly Val Tyr Thr-Arg-Val-Ser-Gln-Tyr-Ile 370 375 380 GAG-TGG-CTG-CAA-AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC 1200Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400CTG CGA GCC CCA TTT CCC TAG 1221Leu Arg Ala Pro Phe Pro 405 <210> 6 <211> 406<212> PRT<213> artificial sequence<220> <223> Aminoacid sequence of recombinant mutantof bloodcoagulation factor VII in which the 164th Cysteine is replaced withAlanine and the 299Valine is replaced with Cysteine.<400> 6Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu IlePhe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 Gln Cys AlaSer Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His LysAsp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 9095 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys HisGlu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130135 140Asn Ala Ser Lys Pro Gln Gly Arg Ile ValGly Gly Lys Val Cys Pro 145 150 155 160Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190 His-Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Val-Leu 195 200 205 Gly-Glu-His-Asp-Leu Ser Glu His Asp Gly-Asp-Glu-Gln-Ser-Arg-Arg 210215 220Val Ala Gln Valle Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235240 His AspIleAla Leu Leu Arg Leu His Gln Pro Val ValLeu Thr Asp 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260265 270Leu Ala Phe Val Arg Phe SerLeu ValSer Gly Trp Gly Gln Leu Leu 275 280 285AspArg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg 290 295 300 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315320 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335Lys Asp Ser Cys Lys Gly AspSer Gly Gly Pro His Ala Thr His Tyr 340 345 350 Arg GlyThr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365Ala ThrVal Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395400 Leu Arg Ala Pro Phe Pro 405 <210> 7<211> 1221<212> DNA<213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu, and cDNA sequence coding thereof.<400> 7GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TC C-CTG-GAG-AGG-GAG 48Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro-Gly-Ser-Leu-Glu Arg Glu 1 5 10 15 TGC-AAG-GAG-GAG-CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys Glu GluGln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr SerAsp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAGAAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCTGCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC

AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAGCGC TCCTGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTGGCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAAAGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AAT GCC AGC AAACCCCAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175 TTGTGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC576Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190 CAC TGT TTC GAC AAA ATCAA G-AAC-TGG-AGG-AAC-CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp-Arg-Asn-Ile Ala Val Leu 195 200 205 GGC-GAG-CAC-GAC-CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTGGCG CAG GTC ATC ATC CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTGCACCAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAGAGGACG 816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCACGGAAAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCCGGCTAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAGGAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CACTAC 1056Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350 CGG GGC ACG TGG TAC CTG ACG GGC ATCGTC AGC TGG GGC CAG GGC TGC 1104Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG GTG TAC ACC AGGGTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380 GAG-TGG-CTG-CAA-AAG CTC-ATG-CGC-TCA-GAG CCA CGC CCA GGA GTC CTC 1200Glu Trp Leu Gln Lys Leu Met-Arg-Ser-Glu-Pro-Arg-Pro-Gly-Val-Leu 385 390 395 400CTGCGA GCC CCA TTT CCC TAG 1221Leu Arg Ala Pro Phe Pro 405 <210> 8 <211> 406<212> PRT <213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu.<400> 8Ala Asn Ala Phe Leu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 Cys LysGlu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser TyrSer Asp Gly Asp 35 4045 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His Lys Asp AspGln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser LysPro Gln Gly Arg Ile ValGly Gly Lys Val Cys Pro 145 150 155 160 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu-Ile-Asn-Thr-Ile Trp Val Val Ser Ala-Ala 180 185 190 His-Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Va-Leu 195 200 205 Gly Glu His Asp Leu Ser Glu His Asp Gly AspGlu Gln Ser Arg Arg210215 220Val Ala Gln Val Ile Ile Pro Ser ThrTyr Asp Arg Lys Thr Leu Asn 225 230 235 240His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265270 LeuAla Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285AspArg Gly Ala Thr Ala Leu GluLeuMet Val LeuAsn Val Pro Arg 290 295 300 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315320 ProAsn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330335 LysAspSer Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365Ala Thr Val Glu His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp Leu GlnLys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400Leu Arg Ala Pro Phe Pro 405 <210> 9 <211> 1206<212> DNA<213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of blood

coagulation factor VII in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys and and cDNA sequence coding.[thereof] <400>
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CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys Glu Glu Gln-Cys-
Ser-Phe Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG CTG TTC TGG
ATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr SerAsp
Gly Asp 35 40 45 CAG TGT GCC TCA AGTCCA TGC CAGAAAT GGG GGC TCC TGC AAG GAC
CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG
TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln SerTyr Ile
Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC AAG GAT GAC
CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys
Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC
AAGCGC TCCTGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105
110 CGG TGC CAC GAG GGG TAC TCT CTG CTGGCA GAC GGG GTG TCC TGC ACA 384Arg
Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA
TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAAAGA 432Pro Thr Val Glu Tyr Pro Cys
Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AAT GCC AGC AAACCCCAA GGC CGA ATT
GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val
Cys Pro 145 150 155 160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG GTG AAT
GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175
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Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190 CAC TGT TTC GAC AAA
ATCAAGAAC TGG AGG AAC-CTG-ATC-GCG-GTG CTG 624His Cys Phe-Asp-Lys-Ile-Lys Asn
Trp Arg Asn Leu-Ile-Ala-Val-Leu 195 200 205 GGC-GAG-CAC-GAC-CTC AGC GAG CAC GAC
GGG GAT GAG CAG AGC CGG CGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln
Ser Arg Arg 210 215 220 GTGGCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC
ACC ACC AAC720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp
Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255CAT GTG GTG CCC CTC
TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816His Val Val Pro Leu Cys Leu Pro
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Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC
CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG
ACC CAG GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG 960Leu Met Thr Gln
Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu 305 310 315 320 TAC ATG TTC TGT GCC GGC
TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG 1008Tyr Met Phe Cys Ala Gly Tyr Ser Asp
Gly Ser Lys Asp Ser Cys Lys 325 330 335 GGGGAC AGT GGA GGC CCA CAT GCC ACC CAC
TAC CGG GGC ACG TGG TAC1056Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp
Tyr 340 345 350 CTG ACG GGC ATC GTC AGCTGGGGC CAG GGC TGC GCA ACC GTG GGC
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Gly Ser Leu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
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Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro
Ala Phe Glu Gly Arg Asn 65 70 75 80 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn
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His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125Pro Thr Val Glu Tyr Pro Cys
Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys
Val Cys Pro 145 150 155 160Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165
170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185190 HisCys Phe
Asp Lys Ile-Lys-Asn-Trp-Arg Asn Leu Ile Ala Val-Leu 195 200 205 Gly-Glu-His-Asp-Leu Ser Glu
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Gly Thr ThrAsn225 230235 240His Asp Ile Ala LeuLeu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu 305 310 315 320Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys 325 330 335 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr 340 345350 LeuThr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His 355 360 365Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys 370 375 380 Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385 390 395400 Pro <210> 11 <211> 1206<212> DNA<213> artificial sequence<220> <223> Amino acid sequence of recombinantmutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys and and cDNA sequence coding. 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asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.<400> 12Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp35 40 45Gln Cys Ala Ser Ser Pro Cys GlnAsn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu GlyArg Asn 65 70 75 80 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 9095 Gly CysGlu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val GluTyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser Lys Pro Gln Gly Arg Ile Val GlyGly Lys Val Cys Pro 145 150 155 160 Lys Gly GluCys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185190 HisCys Phe Asp Lys Ile-Lys-Asn-Trp-Arg Asn Leu Ile Ala Val-Leu 195 200 205 Gly-Glu-His-Asp-Leu Ser Glu His Asp Gly-Asp-Glu-Gln-Ser-Arg-Arg 210 215 220 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr AspArg Lys Thr LeuAsn225 230235 240His Asp Ile Ala LeuLeu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu 305 310 315 320Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys 325 330 335 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr 340 345350 LeuThr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His 355 360 365Phe Gly Val Tyr Thr Arg Val Ser Gin Tyr Ile Glu Trp Leu Gln Lys 370 375 380 Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385 390 395400 Pro

Field

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

Effect

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or FVIIa has clearly high enzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

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Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln
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Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350Arg Gly Thr Trp Tyr Leu Thr Gly
Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 Ala Thr Val GlyHis Phe Gly Val Tyr Thr Arg Val Ser
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CCT GCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly
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TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala
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ATT CTA GAAAAAA AGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130
135 140 AAT-GCC-AGC-AAA-CCC CAA-GGC-CGA-ATT-GTG GGG GGC AAG GTG TGC CCC
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Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG
TTC TCTGAGAGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260
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Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG
CAG TCACCGAAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys
Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC-ATG-TTC-TGT-GCC GGC TAC
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330 335 AAG-GAC-TCC-TGC-AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CACTAC
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TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAGGGC TGC 1104Arg Gly Thr Trp Tyr
Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG
GTG TAC ACC AGG GTC TCC CAG TAC ATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg
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406<212> PRT<213> artificial sequence<220><223> Aminoacid sequence of recombinant mutant of
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[thereof] <400> 9GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG 48AlaAsn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser LeuGlu Arg Glu 1 5 10 15 TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala ArgGlu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GATGGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AATGGG GGCTCC TGC AAG GAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCTGCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACCAAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTGGCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AAT-GCC-AGC-AAA-CCC CAA-GGC-CGA -ATT-GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly-Arg-Ile-Val-Gly-Gly-Lys-Val-Cys-Pro 145 150 155 160AAAGGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTGAAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175 TTGTGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190CAC TGT TTC GAC AAA ATCAAGAAC TGG AGG AAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGCAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTGGCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CACCAGCCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCTGAGAGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATGG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC GAA GGC TCC TACCTGGA AAG ATC ACG GAG 960Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu 305 310 315 320 TAC ATG TTC TGT GCC GGC-TAC-TCG-GAT-GGC AGC AAG GAC TCC TGC AAG 1008Tyr Met

Phe Cys Ala Gly Tyr-Ser-Asp-Gly-Ser-Lys-Asp-Ser-Cys-Lys 325 330 335 GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG TGGTAC 1056Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr 340 345 350 CTGACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTGGGC CAC 1104Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His 355 360 365 TITGGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG 1152Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys 370 375 380 CTCATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TIT 1200Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385 390 395 400 CCC TAG 1206Proes <210> 10 <211> 401<212> PRT<213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 12 aminoacid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.<400> 10Ala Asn Ala Phe Leu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 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 Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC
 CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro
 Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC
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 ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gl y-Ala-Thr-Ala-
 Leu-Glu Leu Met Val Leu Asn-Val-Pro-Arg 290 295 300 CTG-ATG-ACC-CAG-GAC TGC GAA
 GCC TCC TAC CCT GGA AAG ATC ACG GAG 960Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro
 Gly-Lys Ile Thr Glu 305 310 315 320TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC
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 CGA GCC CCA TTT 1200Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385
 390 395 400 CCC TAG 1206Proes <210> 12 <211> 401<212> PRT<213> artificial sequence<220>
 <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5
 amino acid residues from the 235th Valine to 239threonine are replaced with Asp-Arg-Lys-Thr-Leu
 and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-
 Ser-Tyr-Pro-Gly-Lys.<400> 12Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1
 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg
 Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp35 40 45 Gln-Cys-Ala-Ser-Ser Pro Cys Gln Asn Gly-
 Gly-Ser-Cys-Lys-Asp-Gln 50 55 60 Leu-Gln-Ser-Tyr-Ile Cys Phe Cys Leu Pro-Ala-Phe-Glu-Gly-Arg-
 Asn 65 7075 80 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 8590 95Gly Cys
 Glu Gln Tyr Cys Ser Asp His Thr GlyThr Lys Arg Ser Cys 100 105 110 Arg Cys His Glu Gly Tyr Ser
 Leu Leu Ala Asp GlyVal Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile
 Leu Glu Lys Arg 130 135140 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145
 150 155160 Lys GlyGluCys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys
 Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180185 190 His Cys Phe Asp Lys Ile Lys
 Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205GlyGlu His Asp Leu Ser Glu His Asp Gly Asp Glu
 Gln Ser Arg Arg 210 215 220 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn 225
 230 235240 HisAsp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisValVal
 ProLeuCys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala PheVal Arg Phe Ser Leu
 Val Ser Gly Trp Gly Gln Leu Leu 275 280 285AspArg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn
 Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro-Gly-Lys-Ile-Thr Glu 305 310
 315 320 Tyr-Met-Phe-Cys-Ala Gly Tyr Ser Asp Gly-Ser-Lys-Asp-Ser-Cys-Lys 325 330 335 Gly-Asp-
 Ser Gly Gly Pro His Ala Thr HisTyrArg Gly Thr Trp Tyr 340 345350 LeuThr GlyIle Val Ser Trp Gly
 Gln Gly Cys Ala Thr Val Gly His 355 360365Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu
 TrpLeu Gln Lys 370 375 380Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385
 390 395 400Pro

TECHNICAL PROBLEM

[A prior art and the technical problem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.U.SA, 83, p.2412- 2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).

[0003] If the enzyme activity of the FVIIa [itself] is very weak and it combines with the tissue factor

(TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between biparite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique -- respectively -- (1) -- more -- high -- about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [4 / (4) / a thrombus induction of DIC, and] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration. When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [blood coagulation factor] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scanning, 112 Alanine substitution-product *****'s of FVII and the thing which enzyme activity went up in it are only [one], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical problem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

MEANS

[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.

[0013]

[Elements of the Invention] a group similar to a trypsin group -- the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence (drawing 1) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease (drawing 2). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed (drawing 3). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-**) 159Cys from which 159Cys-164Cys combination was cut, and 164Cyses by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-**) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cys, and replacing the 299th ***** (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ***** (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in drawing 3 , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in drawing 3 -- setting -- the serine protease of others [FVII] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine

(310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo-sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example ***** -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ***** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39) replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [a gene] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfests a host cell, and is acquired by refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

EXAMPLE

[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawing, it illustrates in the example [****] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [as long as there is no notice especially the following] transgenics etc. is TAKARA SHUZO, Toyobo, and par ***** applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [Molecular Basis] of Thrombosis and cDNA array well-known at Hemostasis (-- FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCAGGCCCTCAGGCTCCTGCCTCTG) which added SalI site to the array table array number 1 on the basis of written) -- and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCTGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by SalI and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-

"W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>>view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5, and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by Sall and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared (drawing 6). Moreover, about "pVII-6", the gene obtained in drawing 5 using primer ** and ** of a publication is used as mold, and it was obtained by performing PCR further using primer ** and **. Moreover, about "pVII-39", the gene obtained using primer ** and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array.

[0026] The commercial ***** cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>> above-mentioned manifestation vector were chosen by G418 (1mg/ml), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (***** chromium FVII; Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg [ml] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [as opposed to SDS-PAGE or commercial FVII for the purified alteration field], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack *****, equivalent *****, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin;Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start. The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [protein concentration (it measures by the Bradford method)], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [having freezing activity high two to 6 times] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 μg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys-164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys-299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99 を Trypsin 型へ	3,361	685	4,907	245
VII-31	loop170 を Trypsin 型へ	3,589	877	4,093	205
VII-39	loop99+170 を Trypsin 型へ	8,954	773	11,584	579

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C

condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [finishing / refining] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.

[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1 microM It dilutes with 50mMTris -HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible.

Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what **, and showed hydration activity higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

基質名	構造	水解活性/ $\text{mOD}_{405\text{nm}}/\text{min}$		比 31/W
		VIIa-W	VIIa-31	
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
S-2288	H-D-Ile-Pro-Arg	25.8	304.2	12
S-2366	pyro-Glu-Pro-Arg	11.5	267.8	23
S-2238	H-D-Phe-Pip-Arg	11.3	86.6	8
Chromozym X	D-Nile-Gly-Arg	13.0	48.6	4
S-2302	H-D-Pro-Phe-Arg	7.4	38.3	5
S-2765	Z-D-Arg-Gly-Arg	13.3	20.6	2
Chromozym TRY	CBz-Val-Gly-Arg	5.6	28.2	5
S-2444	pyro-Glu-Gly-Arg	1.5	18.9	13
S-2222	Bz-Ile-Glu-Gly-Arg	5.6	16.4	3
S-2403	pyro-Glu-Phe-Lys	0.3	5.9	19

[0032]

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] Drawing showing the primary structure and the alteration site (asterisk) of FVII.

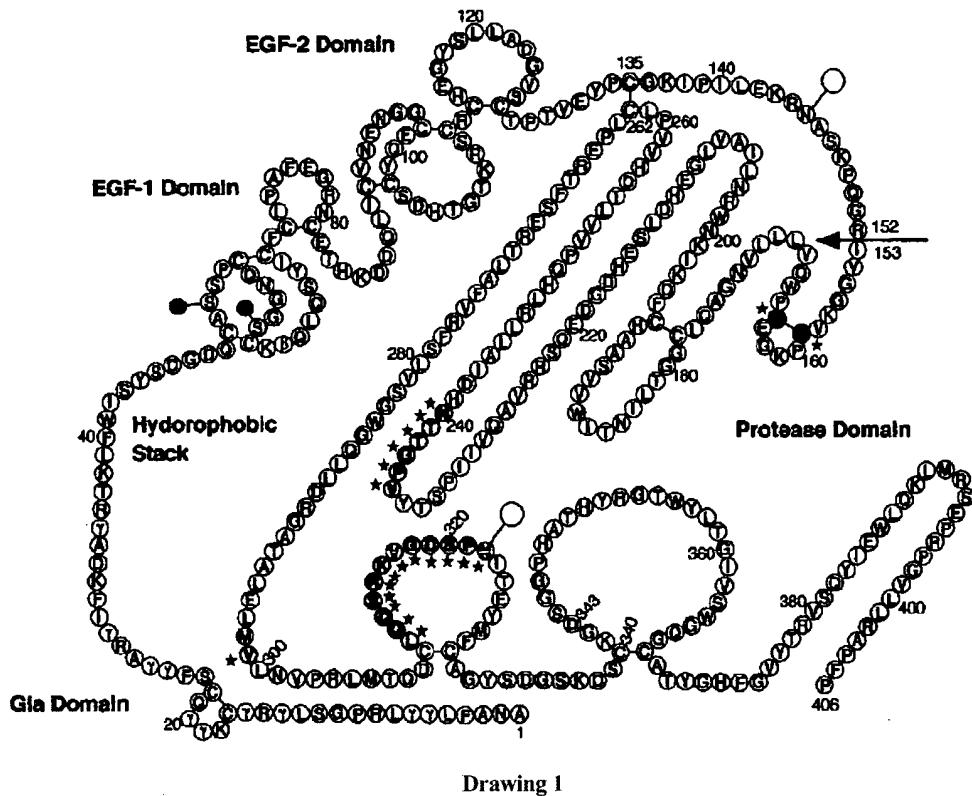
[Drawing 2] Drawing showing the basic structure of the serine protease on the basis of the protease domain amino acid sequence of FVII.

[Drawing 3] Drawing showing the 3D multi-alignment between the various trypsin group serine proteases of X-ray spacial-configuration known.

[Drawing 4] Drawing showing a part of amino acid sequence of wild-type FVII (FVII-Wild) and various FVII alteration fields. This view is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid sequence by the side of the end of N, neither performs an alteration, but it is the same than the 152nd arginine as that of a wild type.

[Drawing 5] Drawing showing the primer array for FVII alteration field production.

[Drawing 6] Drawing showing the construction technique of FVII alteration field manifestation vector.



Drawing 1

153	I V G G K V C P K G E C P W Q V L L L V N G A Q L C G G T I N T I W V V S A A	βストランド1	βストランド2	βストランド3 192	
193	H C F D K I K N W R N L I A V I L G E H D L S E H D G D E Q S R R V A O V T I P S	βストランド4	βストランド5	232	
233	T Y V P G T T N H D I A L L R L H Q P V V L T D H V V P L C L P E R T F S E R T	βストラ	トンド6	272	
273	L A F V R F S L V S G W G Q L L D R G A T A L E L M V L N V P R L M T Q D C L Q	βストラ	トンド7	βストラ	トンド8 312
313	Q S R K V G D S P N I T E Y M F C A G Y S D G S K D S C K G D S G G P H A T H Y	βストラ	トンド9	βストラ	トンド10 352
353	R G T W Y L T G I V S W G Q G C A T V G H F G V Y T R V S Q Y I E W L Q K L M R	βストラ	トンド11	βストラ	トンド12 392
393	S E P R P G V L L R A P F P				

Drawing 2

sequence 1: ヒト血液凝固第VII因子 (pdb ID 1DAN)
 sequence 2: ヒトトリプシン (pdb ID 1TRN)
 sequence 3: ブタ血液凝固第IX因子 (pdb ID 1PFX)
 sequence 4: ウシトリプシン (pdb ID 1TLD)
 sequence 5: ヒト血液凝固第X因子 (pdb ID 1HCG)
 sequence 6: ヒトプロテインC (pdb ID 1AUT)
 sequence 7: ブタカリクリエンA (pdb ID NPKA)
 sequence 8: ウシキモトリブシン (pdb ID 5CHA)
 sequence 9: ブタエラスター^セ (pdb ID 3EST)
 sequence 10: ヒトαトロンビン (pdb ID 1PPB)
 sequence 11: ヒト多形核白血球プロテアーゼ3 (pdb ID 1FUJ)
 sequence 12: ラットトニン (pdb ID 1TON)
 sequence 13: ヒト好中球エラスター^セ (pdb ID 1HNE)
 sequence 14: ヒトウロキナーゼ型プラスミノーゲンアクチベータ (pdb ID 1LMW)
 sequence 15: ヒトカテブシンG (pdb ID 1CGH)
 sequence 16: ラット肥満細胞プロテアーゼ (pdb ID 3RP2)
 sequence 17: ヒト組織型プラスミノーゲンアクチベータ (pdb ID 1RTF)

(図中@位置はすべてのプロテアーゼでのC_α位置が1Å以内で保存されている構造保存部位を示す)



Drawing 3

VII-Wild

IVGGKVC~~P~~KGECPWQVLLL~~VNGAQLCGGT~~INTIWWVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLTDHVPLCLPERTFSERT
LAFVRFSLVSGWGQLLRGATALELMVLPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSC~~K~~GDSGGPHATHYRGTWLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-5

IVGGKVAPKEAPWQVLLL~~VNGAQLCGGT~~INTIWWVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLTDHVPLCLPERTFSERT
LAFVRFSLVSGWGQLLRGATALELMVLPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSC~~K~~GDSGGPHATHYRGTWLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-6

IVGGKVCPKGEAPWQVLLL~~VNGAQLCGGT~~INTIWWVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLTDHVPLCLPERTFSERT
LAFVRFSLVSGWGQLLRGATALELMCLN~~V~~PR~~L~~MTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSC~~K~~GDSGGPHATHYRGTWLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-30

IVGGKVCPKGECPWQVLLL~~VNGAQLCGGT~~INTIWWVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYDRTNHDIALRLHQPVVLTDHVPLCLPERTFSERT
LAFVRFSLVSGWGQLLRGATALELMVLPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSC~~K~~GDSGGPHATHYRGTWLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-31

IVGGKVCPKGECPWQVLLL~~VNGAQLCGGT~~INTIWWVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTNHDIALRLHQPVVLTDHVPLCLPERTFSERT
LAFVRFSLVSGWGQLLRGATALELMVLPRLMTQDCEASYP-----GKITEYMFCAGY
SDGSKDSC~~K~~GDSGGPHATHYRGTWLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-39

IVGGKVCPKGECPWQVLLL~~VNGAQLCGGT~~INTIWWVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYDRTNHDIALRLHQPVVLTDHVPLCLPERTFSERT
LAFVRFSLVSGWGQLLRGATALELMVLPRLMTQDCEASYP-----GKITEYMFCAGY
SDGSKDSC~~K~~GDSGGPHATHYRGTWLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

(下線部は改変部位を表す)

①VII-PWN Sense ; 5'-GGGGTCGACATGGTCTCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3'
Factor VII Wild type のシグナル配列からのプライマーデザイン
5'-GGGGTCGACATGGTCTCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3'
Sali M V S Q A L R L L C L L

②VII-PWC Antis ; 5'-CCCGGATCCCAGGGAAATGGGGCTCGCAGGAGGACTCCTGGCG-3'
Factor VII Wild type のカルボキシ末端までのプライマーデザイン
5'-CCCGGATCCCAGGGAAATGGGGCTCGCAGGAGGACTCCTGGCG-3'
BamHI

③VII-P5-1 Sense ; 5'-ATTGTGGGGCAAGGTGGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'
④VII-P5-2 Antis ; 5'-GACCTGCCATGGGGCTCCCTTGGGGCCACCTTGCCCCCAAT-3'
VII-5のプライマーデザイン (C159A, C164A)
5'-ATTGTGGGGCAAGGTGGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'
3'-TAACACCCCCCGTCCACCGGGGTTCCCTCCGGGTACCGTCCAG-5'
I V G G K V A P K G E A P W Q V

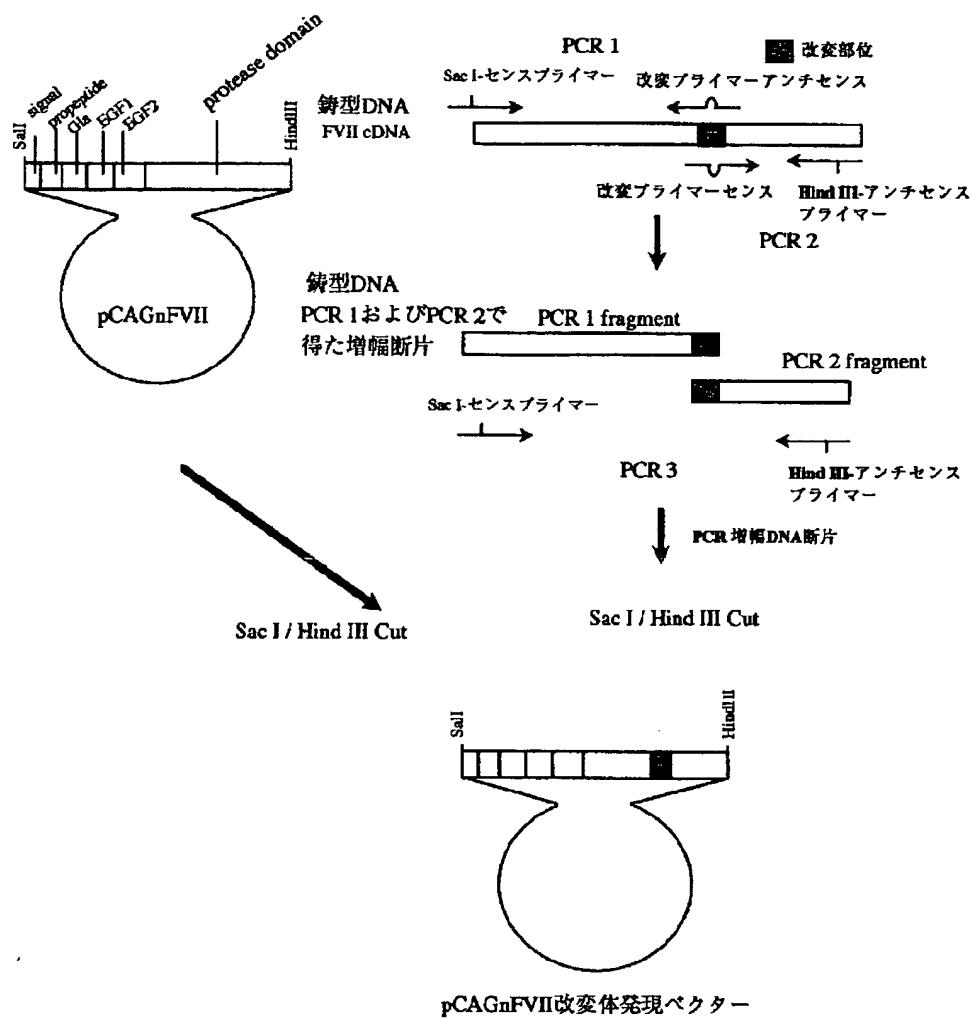
⑤VII-P6-1 Sense ; 5'-TGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'
⑥VII-P6-2 Antis ; 5'-GACCTGCCATGGGGCTCCCTTGGGCA-3'
VII-6のプライマーデザイン① (C164A)
5'-TGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'
3'-ACGGGTTCCCTCCGGGTACCGTCCAG-5'
C P K G E A P W Q V

⑦VII-P6-3 Sense ; 5'-CTGGAGCTCATGTGCCCTAACGTGCCCGG-3'
⑧VII-P6-4 Antis ; 5'-CCGGGGCACGTTGAGGCACATGAGCTCAG-3'
VII-6のプライマーデザイン② (v299C)
5'-CTGGAGCTCATGTGCCCTAACGTGCCCGG-3'
3'-GACCTCGAGTACCGGAGTTGCACGGGCC-5'
L E L M C L N V P R

⑨VII-P30-1 Sense ; 5'-ATCCCCAGCACGTACGACAGGAAGACTCTGAACACGACATCGCGCTG-3'
⑩VII-P30-2 Antis ; 5'-CAGCGCGATGTCGTGCTCAAGTCTCTGTGCTACGTGCTGGGAT-3'
VII-30のプライマーデザイン (VPGTTN→DRKTLN)
5'-ATCCCCAGCACGTACGACAGGAAGACTCTGAACACGACATCGCGCTG-3'
3'-TAGGGTGTGCGATGTCGTGCTCTGAGACTTGGTGTAGCGCGAC-5'
I P S T Y D R K T L N H D I A L

⑪VII-P31-1 Sense ; 5'-ATGACCCAGGACTGCCAAGGCCCTACCCCTGGAAAGATCACGGAGTACATG-3'
⑫VII-P31-2 Antis ; 5'-CATGTACTCCGTGATCTTCCAGGTAGGAGGCTTCGAGTCCTGGTCAT-3'
VII-31のプライマーデザイン (LQOSRKVGDSPN→EASYPGR)
5'-ATGACCCAGGACTGCCAAGGCCCTACCCCTGGAAAGATCACGGAGTACATG-3'
3'-TACTGGGTCCGTGCGCTCGGAGGATGGGACCTTCTAGTGCCTCATGTAC-5'
M T Q D C E A S Y P G K I T E Y M

Drawing 5



pCAGnFVII 改変体発現ベクター

Drawing 6

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(54)【発明の名称】 血液凝固第VII因子改変体

(57)【要約】

【課題】 酵素活性を増強させた血液凝固第VII因子(以下、FVII)及び/または活性化型血液凝固第VII因子(以下、FVIIa)の改変体を作製する。

【解決手段】 下記から選択される少なくとも一つの改変を含むことを特徴とするFVII/FVIIa改変体及び当該改変体を有効成分として含有する医薬品組成物。 (a) FVIIの159Cys-164Cysの切断、(b) FVII内の、233番目のスレオニン(233Thr)から240番目のアスパラギン(240Asn)のアミノ酸配列からなるループ構造を構成するアミノ酸配列またはその一部を、置換、追加または削除、(c) FVII内の、304番目のアルギニン(304Arg)から329番目のシスティン(329Cys)の介在アミノ酸配列を構成するアミノ酸配列またはその一部を、置換、追加または削除。

【特許請求の範囲】

【請求項1】 下記から選択される少なくとも一つの改変を含むことを特徴とする血液凝固第VII因子(以下、FVII)または活性化型血液凝固第VII因子(以下、FVIIa)の改変体。

(a) FVII内の、159番目のシステイン(159Cys)と164番目のシステイン(164Cys)からなるジスルフィド結合(159Cys-164Cys)を切断する。

(b) FVII内の、233番目のスレオニン(233Thr)から240番目のアスパラギン(240Asn)のアミノ酸配列からなるループ構造(以下、99-1oopと称することもある)を構成するアミノ酸配列またはその一部を、置換、追加または削除する。

(c) FVII内の、304番目のアルギニン(304Arg)から329番目のシステイン(329Cys)の介在アミノ酸配列を構成するアミノ酸配列またはその一部を、置換、追加または削除する。

【請求項2】 上記159Cysと164CysをCys以外のアミノ酸残基によって置換することにより、当該159Cys-164Cysが切断されることを特徴とする請求項1に記載の改変体。

【請求項3】 配列表配列番号4記載のアミノ酸配列からなる請求項1または2に記載の改変体。

【請求項4】 164CysをCys以外のアミノ酸残基によって置換し、かつ、299番目のバリン(299Val)をCysに置換することにより、159Cys-164Cysが切断され、かつ159Cysと299Cys間ににおいてジスルフィド結合(159Cys-299Cys)が形成されることを特徴とする請求項1に記載の改変体。

【請求項5】 配列表配列番号6記載のアミノ酸配列からなる請求項1または4に記載の改変体。

【請求項6】 FVIIの99-1oopのアミノ酸配列が、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換されることを特徴とする請求項1に記載の改変体。

【請求項7】 他のトリプシン族セリンプロテアーゼがヒトトリプシンである請求項1または6に記載の改変体。

【請求項8】 FVIIの99-1oop内の235番目のバリン(235Val)から239番目のスレオニン(239Thr)までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるAsp-Arg-Lys-Thr-Leuで置換されることを特徴とする請求項1、6または7に記載の改変体。

【請求項9】 配列表配列番号8記載のアミノ酸配列からなる請求項1または6から8のいずれかに記載の改変体。

【請求項10】 FVII内の、304番目のアルギニン(304Arg)から329番目のシステイン(329Cys)

s)の介在アミノ酸配列を構成するアミノ酸配列またはその一部が、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換されることを特徴とする請求項1に記載の改変体。

【請求項11】 他のトリプシン族セリンプロテアーゼがヒトトリプシンである請求項1または10に記載の改変体。

【請求項12】 FVII内の、310番目のシステイン(310Cys)から329番目のシステイン(329Cys)の介在アミノ酸配列(以下、170-1oopと称することもある)を構成するアミノ酸配列またはその一部が、置換、追加または削除される請求項1、10または11に記載の改変体。

【請求項13】 FVIIの170-1oop内の311番目のロイシン(311Leu)から322番目のアスパラギン(322Asn)までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Lysで置換されることを特徴とする請求項1または10から12のいずれかに記載の改変体。

【請求項14】 配列表配列番号10記載のアミノ酸配列からなる請求項1または10から13のいずれかに記載の改変体。

【請求項15】 FVIIの99-1oop内の235番目のバリン(235Val)から239番目のスレオニン(239Thr)までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるAsp-Arg-Lys-Thr-Leuで置換され、かつ、170-1oop内の311番目のロイシン(311Leu)から322番目のアスパラギン(322Asn)までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Lysで置換することを特徴とする請求項1に記載の改変体。

【請求項16】 配列表配列番号12記載のアミノ酸配列からなる請求項1または15に記載の改変体。

【請求項17】 請求項1から16のいずれかに記載の改変体を有効成分として含有する医薬品組成物。

【請求項18】 請求項17の医薬品組成物からなる血友病インヒビター患者の治療に有効な薬剤。

【発明の詳細な説明】**【0001】**

【発明の属する技術分野】 本願発明は、酵素活性を増強させた血液凝固第VII因子(以下、FVIIと称することがある)及び/または活性化血液凝固第VII因子(以下、FVIIaと称することがある)の改変体に関するものである。詳細には、本願発明は、FVIIに特有なアミノ酸配列を、置換・欠損することにより、活性が増強されたFVII/FVIIa改変体、当該改変体を有効成分として含有する医薬品組成物、及び当該医薬品組成物を用いた治療方法に関するものである。

品組成物からなる血友病インヒビター患者の治療に有効な薬剤に関するものである。

【0002】

【従来の技術および解決すべき課題】FVIIはビタミンK依存性の血液凝固因子であり、外因系血液凝固の開始因子であることは広く知られている。他のビタミンK依存性凝固因子と同様にN末端から35残基までのアミノ酸配列に10個のγカルボキシグルタミン酸（以下、Glaと称することがある）からなるGla領域を有している（Proc.Natl.Acad.Sci.U.S.A., vol.83, p.2412-2416, 1986）。FVIIは、in vitroにおいて、活性化血液凝固第X因子（以下、FXaと称することがある）、活性化血液凝固第IX因子（以下、FIXaと称することがある）またはトロンビン（以下、FIIaと称することがある）によって、152Arg-153Ileが加水分解され、一個のS-S結合で架橋されたH鎖とL鎖から構成される活性型FVIIすなわちFVIIaに変換されるることは知られている（J.Biol.Chem., vol.251, p.4797-4802, 1976）。

【0003】FVIIa自体の酵素活性は極めて弱く、補酵素である組織因子（TF）と結合すると劇的に上昇する（Komiyama et al., Biochemistry, 29(40), pp.9418-25(1990)）。FVIIaとTFの1次構造、その複合体の結晶構造、さらに両分子間の結合部位もアミノ酸残基レベルで判明しているが、その触媒活性増幅機構の詳細（TF結合に伴う立体構造変化）は依然として不明である（Banner et al., et al., Nature 380(6569): pp.41-6(1996)）。

【0004】血友病A及び血友病B患者に対する補充療法として、血液凝固第VIII因子（以下、FVIIIと称することがある）及び血液凝固第IX因子（以下、FIXと称することがある）製剤の投与が行なわれている。しかし、当該治療法に伴いFVIII及びFIXに対する中和抗体（インヒビターと呼ばれることがある）の出現が問題視されている。

【0005】このようなインヒビターを生じた血友病患者の対処療法として、(1) FVIII因子の過剰投与、(2) プタFVIII因子の投与、(3) FIX、FVII、FIX及びFXからなる複合体製剤の投与、(4) FVIIa製剤の投与などがある。しかしながらこれらの方法は、それぞれ(1)については、より高力価なインヒビターの誘導による症状悪化、(2)については、抗原性によるショック、(3)については、血栓・DICの誘発、(4)については、治療効果が不十分であることや大量・頻回投与によりコストが高いなどの問題を抱えている。これらの中で、効果と危険性のバランスを考慮した場合、最も効率的なものは(4)のFVIIa製剤の投与である。しかしながら、FVIIa製剤はその活性の弱さのため、止血効果を發揮するには、前述したように大量投与と頻回投

与を必要とし、治療コストを大きく高めている。また、その治療効果も血友病患者に対して行われている従来の補充療法に比べれば充分とはいえない。

【0006】この問題を解決するための手段として、酵素活性を上昇させたFVIIの改変体を作製することが挙げられるが、これは一般的に困難であることが知られている（タンパク質の構造入門、勝部幸輝監修、教育社発行、1992年）。特に、血液凝固因子について、以下の理由により改変による活性増強は困難と考えられている。

【0007】血友病は血液凝固因子の異常であるが、量的欠損に伴う活性低下と質的異常による活性低下の2つに分類される。このうち質的異常の多くは（ポイント）ミューテーションであることが知られており、FIXの異常である血友病Bの患者の解析が行われた結果、FIXの構造全域にわたって分子異常が存在することが明らかとなり、中にはたった1個のアミノ酸が置換されただけで、活性が1%以下になる例が多数ある。従って、血液凝固因子についてむやみに改変を行っても、活性低下を招くのは明らかである。

【0008】また、Alanine Scanningで得られた情報（Dickinson et al., Proc. Natl. Acad. Sci. USA, 93(25), pp.14379-84(1996)）によれば、FVIIの112個のAlanine置換体について、その中で酵素活性が上がったものは唯一1つであり、しかもその規則性は見いだされていない。

【0009】その他の試みとして、Illopferらは、FIXを構成する一部のドメインの数アミノ酸残基から構成される構造単位を欠損・置換する方法を用い、合成基質活性を上昇させたFIXフラグメント改変体を作製した（EMBO J., 16(22), pp.6626-35(1997)）。しかしながら、これはインタクトなFIXではなく、FIXの部分フラグメントを大腸菌で発現させたもので合成基質活性を見ているに過ぎないため、血液凝固活性を増強しうるどころか血液凝固活性すら有さないものである。さらに、これはFIXに関するものであり、構造も特異性も全く異なる別物質であるFVIIに対して何ら示唆するものではなく、FVIIの酵素活性を増強させた改変体についても、これまで何ら報告はない。

【0010】このように、強い酵素活性を有する改変体の作製は、特に血液凝固因子においては困難と考えられていた。FIXにおいて、その部分フラグメントについて合成基質活性を上げる試みはなされたものの、インタクトな分子として高い酵素活性を有する血液凝固因子の改変体についてはこれまで報告例はない。

【0011】従って、本発明の解決すべき課題は、一般に血液凝固因子の改変は困難と考えられている状況において、血友病インヒビター患者の治療に有効な強い活性を有するFVII及び/またはFVIIaを作製・提供することである。

【0012】

【課題を解決するための手段】上記のような状況において、本発明者らは、それ自身高い酵素活性を有するFVIIを作製すべく鋭意研究を重ね種々の検討を行った結果、本願発明を完成するに至った。本願発明は、FVIIと各種セリンプロテアーゼとアミノ酸配列構造を比較し、FVIIに特有のアミノ酸配列部位を明確にし、その特有な部位を、欠損・置換することにより、活性が増強されたFVII及び/またはFVIIa改変体を作製することに成功したものである。

【0013】

【発明の構成】トリプシン族に類する一群のセリンプロテアーゼの基本構造は、約250残基からなり、アミノ酸配列上でおよそ、その前半と後半の2つのドメインに分けられる(図1)。各ドメイン内にはそれぞれ6本の β ストランドがあり、プロテアーゼとして計12本の β ストランドを有する構造で形成されている(図2)。これら12本の β ストランドはいわばセリンプロテアーゼの骨格構造となっており、各ストランド間をつなぐループないし、ヘリックス領域が、その基質特異性やコファクターとの反応性などのプロテアーゼ活性を担っていると考えられている。セリンプロテアーゼの例としては、FIII、FVII、FVIII、FIX、FX等の各種血液凝固因子、プラスミン等の血栓溶解酵素、またはトリプシン、キモトリプシン、エラスターーゼなどの消化酵素がある。そこで、FVIIをはじめとする各種セリンプロテアーゼのアミノ酸配列構造の比較を行い、FVIIに特徴的な領域を特定した(図3)。そして、これらの部位を改変のターゲットとし、他のセリンプロテアーゼの構造を参考に、FVIIのアミノ酸配列を欠損・置換することによって、高い酵素活性を有するFVII改変体を作製した。これらの改変体について詳細に説明する。

【0014】(a) 159Cys-164Cys結合が切断された改変体

(a-①) 159Cysと164CysをCys以外のアミノ酸残基によって置換することにより、当該159Cys-164Cysが切断された改変体(VII-5)。この改変体の具体例として、Cysをそれぞれアラニン(Ala)に置換したものを配列表配列番号3または4に記載した。ここで、置換に用いるCys以外のアミノ酸残基の一例として、Alaを選択したが、置換によって、Cys-Cys結合を切断すること以外に酵素活性を失活させるなどの大きな障害を与えない限り、任意のアミノ酸が選択可能である。

(a-②) 164CysをCys以外のアミノ酸残基によって置換し、かつ、299番目のヴァリント(299Val)をCysに置換することにより、159Cys-164Cysが切断され、かつ159Cysと299Cys間ににおいてジスルフィド結合(159Cys-299Cys)

が形成された改変体(VII-6)。この改変体の具体例として、Cys以外のアミノ酸残基としてAlaを用いて置換したものを配列表配列番号5または6に記載した。ここで、置換に用いるCys以外のアミノ酸残基については上述の通り、置換によって159Cys-164Cys結合を切断すること以外に酵素活性を失活させるなどの大きな障害を与えない限り、Ala以外の他のアミノ酸が選択可能である。

【0016】(b) FVII内の、233番目のスレオニン(233Thr)から240番目のアスパラギン(240Asn)のアミノ酸配列からなるループ構造(以下、99-1oopと称することもある)を構成するアミノ酸配列またはその一部が、置換、追加または削除された改変体。

この領域は、図3に示すようにセリンプロテアーゼに共通に存在する β ストランド5と β ストランド6の間に介在するアミノ酸配列を含むものである。この領域を他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換することが好ましい。トリプシン族セリンプロテアーゼの好適な一例として、ヒトトリプシンが挙げられる。さらに、具体的な例として、FVIIの99-1oop内の235番目のバリン(235Val)から239番目のスレオニン(239Thr)までのアミノ酸配列が、トリプシンのループ構造内にあるAsp-Ala-Lys-Thr-Leuで置換された改変体(VII-30)が挙げられる。この改変体を配列表配列番号7または8に記載した。

【0017】(c) FVII内の、304番目のアルギニン(304Arg)から329番目のシステイン(329Cys)の介在アミノ酸配列を構成するアミノ酸配列またはその一部が、置換、追加または削除された改変体。

特にこの領域は、図3に示すように、セリンプロテアーゼに共通に存在する β ストランド8と β ストランド9の間に介在するアミノ酸配列において、FVIIは他のセリンプロテアーゼと比較して数アミノ酸残基長いという特徴を有することから、FVII改変における好適なターゲットとなりうるものと推測される。この領域を、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換することが好ましい。トリプシン族セリンプロテアーゼの好適な一例として、ヒトトリプシンが挙げられる。また、FVII内の置換、追加、削除しうる好ましい領域は、310番目のシステイン(310Cys)から329番目のシステイン(329Cys)のアミノ酸配列からなるループ構造(170-1oopと称することもある)を構成するアミノ酸配列またはその一部である。さらに、具体的な例として、FVIIの170-1oop内の311番目のロイシン(311Leu)から322番目のアスパラギン(322Asn)までのアミノ酸配列が、ヒトトリプシンのループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Ly

sで置換された改変体（VII-31）が挙げられる。この改変体を配列表配列番号9または10に記載した。

【0018】さらに、上記（a）から（c）の改変を適宜組み合わせることも可能である。その具体例として、例えば、（b）と（c）の組み合わせ、すなわち、FVIIの99-100p内の235番目のバリン（235V-al）から239番目のスレオニン（239T-hr）までのアミノ酸配列が、ヒトトリプシンのループ構造内にあるAsp-Arg-Lys-Thr-Leuで置換され、かつ、170-100p内の311番目のロイシン（311Leu）から322番目のアスパラギン（322Asn）までのアミノ酸配列が、トリプシンのループ構造内にあるGlu-Ala-Ser-Tyr-Pro-Gly-Lysで置換された改変体（VII-39）が挙げられる。この改変体を配列表配列番号11または12に記載した。

【0019】上述した改変体は、遺伝子組換え法を用いて得ることができる。発現宿主としては、動物細胞等の真核細胞が好ましい。本発明の改変体は、上記各改変体のアミノ酸配列をコードするcDNAを適当な発現ベクターに組み込み、宿主細胞にトランسفクトし、目的の遺伝子を発現している細胞をクローニングし、得られた安定発現株を培養後、精製することにより得られる。

【0020】本願発明のFVII改変体は各種化学処理等を行い、活性化型FVII（FVIIa）改変体として使用することができる。

【0021】本願発明のFVII/FVIIa改変体は、治療、診断または他の用途のために製薬学的調合剤に処方することができる。静脈内投与のための調合剤に対しては、組成物を、通常、生理学的に適合しうる物質、例えば塩化ナトリウム、グリシン等を含み、かつ生理学的条件に適合しうる緩衝されたpHを有する水溶液中に溶解する。また、長期安定性の確保の観点から、最終的剤型として凍結乾燥製剤の形態をとることも考慮されうる。なお、静脈内に投与される組成物のガイドラインは政府の規則、例えば「生物学的製剤基準」によって確立されている。本願発明のFVII/FVIIa改変体からなる医薬品組成物の具体的な用途としては、FVIIまたはFIXの補充療法により当該血液凝固因子に対してインヒビターを生じた血友病インヒビター患者の治療が挙げられる。

【0022】

【実施例】本願発明を実施例により例示するが、これら実施例は本願発明を限定するものではない。本願発明について添付図面を参照して特定な実施例にて例示する。実施例は改変体を動物細胞（CHO-K1）の培養上清中に発現させたものである。以下特に断りが無い限り、遺伝子組換えに関わる試薬等は、宝酒造、東洋紡、パーキンエルマーアプライドNew England Biolabs社の製品を用いた。

【0023】《実施例1. FVII cDNAのクローニング》ヒト肝臓cDNAライブラリー（宝酒造）を購入し、文献等（Molecular Basis of Thrombosis and Hemostasis）で公知のcDNA配列（配列表配列番号1に記載）を基にSalIサイトを付加したFVII合成DNAセンスプライマー（VII-PWN；GGGGTCGACATGGCTCCAGGCCCTCAGGCTCTGCCTCTG）及び、BamHIサイトを付加したアンチセンスプライマー（VII-PWC；CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGCG）を用いてPCRを行い、市販のクローニングベクターpCRII（Invitrogen社）にクローニングした。この際、常法によりDNAシークエンスを行い、文献等で公知の配列（Hagen FS et al., PNAS 1986; 83; 2412-6）を有することを確認した。

【0024】《実施例2. FVII発現ベクターの調製》発現ベクターpCAGn（特許第2824434号公報）をSalI、BamHIで消化し、そこにFVIIをコードした配列を含む上記実施例1で調製したDNAフラグメントをSalI、BamHIでカットしたものをライゲーションし、大腸菌JM105に形質転換し、アンピシリン含有のLB寒天培地上で培養し、形質転換大腸菌を選択した。出現したコロニーを市販の培地で一晩培養し、目的の発現プラスミドを抽出精製し「pVII-W」を調製した。この発現ベクターのDNAシークエンスを行い、目的の遺伝子配列を有することを確認した。

【0025】《実施例3. 改変体発現ベクターの調製》図4に示すアミノ酸配列を有する各FVII改変体を、以下の方法で作成した。なお図4は、FVIIの153番目のイソロイシンよりC末側のアミノ酸配列のみ示したもので、152番目のアルギニンよりN末側のアミノ酸については、いずれも改変は行っておらず野生型と同じである。図5に示す合成DNAプライマーを用いてFVII遺伝子を鋳型としてPCRを行いそれぞれの增幅断片を得る。各增幅断片と、発現ベクターpCAGnをSalI及びBamHIでカットしたものをライゲーションし、大腸菌JM105に形質転換し、アンピシリン含有のLB寒天培地上で培養し、形質転換大腸菌を選択した。出現したコロニーを市販の培地で一晩培養し、目的の発現プラスミドを抽出精製し「pVII-5」、「pVII-30」、及び「pVII-31」を調製した（図6）。また、「pVII-6」については、図5に記載のプライマー⑤及び⑥を用いて得られた遺伝子を鋳型にし、さらにプライマー⑦及び⑧を用いてPCRを行うことにより得られた。また、「pVII-39」については、プライマー⑨及び⑩を用いて得られた遺伝子を鋳型にし、さらにプライマー⑪及び⑫を用いてPCRを行うことにより得られた。さらにDNAシークエンスを行い、これらのプラスミドが目的の配列を有することを確認した。

【0026】《実施例4. 各改変体の培養上清への発現及び精製》上記発現ベクターを、市販のリポフェクチン

試薬でCHO細胞に対して形質導入を行い、G418(1 mg/ml)で選択し、目的の遺伝子を発現している細胞を限外希釈法によりクローニングした。FVII改変体の発現の確認は、市販のFVIIに対するELISAキット(アセラクロムFVII; Diagnostica Strago社)で行った。得られた安定発現株を無血清培地(ASF104、味の素、ペニシリン、ストレプトマイシン、20 µg/mlビタミンK、1 mM酪酸)で培養し、抗ヒトFVIIモノクローナル抗体カラムで精製した(特許第2824430号公報)。平衡化・洗浄及び溶出は、平衡化・洗浄バッファー(50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, 2 mM Ca²⁺)、溶出バッファー(50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, 10mM EDTA)を用いて行った。純化された改変体をSDS-PAGE、または市販のFVIIに対する抗体を用いて、ウエスタンプロットを行い、FVII改変体であることを確認した。

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 µg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys→164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys→299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99をTrypsin型へ	3,361	685	4,907	245
VII-31	loop170をTrypsin型へ	3,589	877	4,093	205
VII-39	loop99+170をTrypsin型へ	8,954	773	11,584	579

【0029】《実施例6. 活性化された各改変体の調製》精製した各改変体を、50mM Tris, pH 7.45, 0.1M NaClに透析し、FXaを1/100(モル比)加え、50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100 µg/mlリソ脂質(Platerin(登録商標) Organotecnica社)、10mM Ca²⁺、37°Cの条件下、1~60分でインキュベーションし活性化した。活性化後、50mM Benzamidine-HClを加えて反応を停止し、抗ヒトFVIIモノクローナル抗体カラムで精製した(実施例4と同じ方法)。精製済みの各活性化改変体はTBS pH 8.0(0.1% PEG 8000含有)に透析し、-80°Cに凍結保存した。活性化の程度は、SDS-PAGEで確認した。

【0030】《実施例7. 活性化された各改変体の合成基質に対する水解活性測定》実施例6に従い活性化され

た。

【0027】《実施例5. 各改変体の凝固活性の測定》各改変体の凝固活性は常法に従い、FVII欠乏血漿を用いた凝固法で測定した。精製した各改変体を50~5 ng/mlになるようにTris-BSAで希釈し、FVII欠乏血症と等量混ぜ、37°Cで3分加温後、再脂質化TF(トロンボプラスチン; Dade社)を等量添加し、凝固反応を開始させた。凝固時間を測定し、標準曲線と希釈率より凝固活性を求めた。凝固活性を蛋白濃度(Bradford法で測定)当たりに換算し比活性を求めた結果を表1に記す。その結果、本発明のFVII改変体は、血漿由來FVII及び野生型組換えFVIIと比較して、2~6倍高い凝固活性を有することが明らかとなった。

【0028】

【表1】

た改変体VIIa-31を0.1 µMになるまで50mM Tris-HCl, 100mM NaCl, 10mM Ca²⁺, 0.1% PEG 8000, pH 8.0で希釈し、そこに種々の合成基質を最終濃度1.0mMになるように加え、最終容量を200 µlとし、30°Cで反応させ、1分間当たりの基質の水解量を見た。温度制御が可能な microplate reader Spectra max plus (Molecular device社)でpNAの遊離を405nmによる発色度として測定した。この結果を表2に示す。本発明の改変体の一つであるVIIa-31は、何れの合成基質を対しても野生型(VIIa-W)より高い水解活性を示し、その範囲は2~23倍であった。

【0031】

【表2】

基質名	構造	水解活性 / mOD _{405nm} /min		比 31/W
		VIIa-W	VIIa-31	
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
	H-D-Ile-Pro-Arg	25.8	304.2	12
	pyro-Glu-Pro-Arg	11.5	267.8	23
	H-D-Phe-Pip-Arg	11.3	86.6	8
Chromozym X	D-Nile-Gly-Arg	13.0	48.6	4
	H-D-Pro-Phe-Arg	7.4	38.3	5
	S-2302	13.3	20.6	2
	Z-D-Arg-Gly-Arg	5.6	28.2	5
Chromozym TRY	CBz-Val-Gly-Arg	1.5	18.9	13
	pyro-Glu-Gly-Arg	5.6	16.4	3
	Bz-Ile-Glu-Gly-Arg	0.3	5.9	19
	pyro-Glu-Phe-Lys			

【0032】

【発明の効果】このように本願発明により得られたFV

II及び／またはFVIIaの改変体は、野生型のFVIIに比べて明らかに高い酵素活性を有するものである。従って、本願発明の改変体は、血友病インヒビター

患者への補充療法として極めて有効な薬剤となりうるものである。

【配列表】

SEQUENCE LISTING

<110> The Chemo-Sero-Therapeutic Research Institute

<120> Recombinant mutants of blood coagulation factor VII

<160> 12

<210> 1

<211> 1221

<212> DNA

<213> blood coagulation factor VII

<400> 1

GCC AAC GCG TTC CTG GAG GAG CTG CCG CCG GGC TCC CTG GAG AGG GAG	48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu	
1 5 10 15	
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG	96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys	
20 25 30	
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC	144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp	
35 40 45	
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG	192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln	
50 55 60	
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC	240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn	
65 70 75 80	
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC	288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly	
85 90 95	
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT	336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys	
100 105 110	
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA	384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr	
115 120 125	
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA	432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg	
130 135 140	
AAT GCC AGC AAA CCC CAA CGC CGA ATT GTG GGG GGC AAG GTG TGC CCC	480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro	
145 150 155 160	
AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG	528
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln	
165 170 175	
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC	576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala	
180 185 190	
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG	624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu	

195	200	205	
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg			
210	215	220	
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn			
225	230	235	240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp			
245	250	255	
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr			
260	265	270	
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu			
275	280	285	
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg			
290	295	300	
CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser			
305	310	315	320
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser			
325	330	335	
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr			
340	345	350	
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys			
355	360	365	
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile			
370	375	380	
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu			
385	390	395	400
CTG CGA GCC CCA TTT CCC TAG			
1221			
Leu Arg Ala Pro Phe Pro			
405			
<210> 2			
<211> 406			
<212> PRT			
<213> blood coagulation factor VII			
<400> 2			
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu			
1	5	10	15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys			
20	25	30	
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp			

35	40	45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln		
50	55	60
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn		
65	70	75
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly		
85	90	95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys		
100	105	110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr		
115	120	125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg		
130	135	140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro		
145	150	155
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln		
165	170	175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala		
180	185	190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu		
195	200	205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg		
210	215	220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn		
225	230	235
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp		
245	250	255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr		
260	265	270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu		
275	280	285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg		
290	295	300
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser		
305	310	315
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser		
325	330	335
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr		
340	345	350
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys		
355	360	365
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile		
370	375	380
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu		
385	390	395
Leu Arg Ala Pro Phe Pro		
405		
<210> 3		
<211> 1221		
<212> DNA		

<213> artificial sequence

<220>

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which both of the 159th Cysteine and the 164th Cysteine are replaced with Alanine, and cDNA sequence coding thereof.

<400> 3

GCC AAC GCG TTC CTG GAG GAG CTG CCG CCG GGC TCC CTG GAG AGG GAG	48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu	
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TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG	96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys	
20 25 30	
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC	144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp	
35 40 45	
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG	192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln	
50 55 60	
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC	240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn	
65 70 75 80	
TGT GAG ACC CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC	288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly	
85 90 95	
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT	336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys	
100 105 110	
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA	384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr	
115 120 125	
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA	432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg	
130 135 140	
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG GCC CCC	480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro	
145 150 155 160	
AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG GTG AAT GGA GCT CAG	528
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln	
165 170 175	
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC	576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala	
180 185 190	
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG	624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu	
195 200 205	
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG	672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg	
210 215 220	
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC GCG GGC ACC ACC AAC	720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn	

225	230	235	240	
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC				768
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp				
245	250	255		
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG				816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr				
260	265	270		
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG				864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu				
275	280	285		
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG				912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg				
290	295	300		
CTG ATG ACC CAG GAC TGC CTG CAG TCA CGG AAG GTG GGA GAC TCC				960
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser				
305	310	315	320	
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC				1008
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser				
325	330	335		
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC				1056
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr				
340	345	350		
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC				1104
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys				
355	360	365		
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC				1152
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile				
370	375	380		
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC				1200
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu				
385	390	395	400	
CTG CGA GCC CCA TTT CCC TAG				1221
Leu Arg Ala Pro Phe Pro				
405				
<210> 4				
<211> 406				
<212> PRT				
<213> artificail sequence				
<220>				
<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which both of the 159th Cysteine and the 164th Cysteine are replaced with Alanine.				
<400> 4				
Ala Asn Ala Phe Leu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu				
1	5	10	15	
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys				
20	25	30		
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp				
35	40	45		
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln				

50	55	60
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn		
65	70	75
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly		
85	90	95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys		
100	105	110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr		
115	120	125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg		
130	135	140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro		
145	150	155
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln		
165	170	175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala		
180	185	190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu		
195	200	205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg		
210	215	220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn		
225	230	235
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp		
245	250	255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr		
260	265	270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu		
275	280	285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg		
290	295	300
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser		
305	310	315
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser		
325	330	335
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr		
340	345	350
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys		
355	360	365
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile		
370	375	380
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu		
385	390	395
Leu Arg Ala Pro Phe Pro		
405		
<210> 5		
<211> 1221		
<212> DNA		
<213> artificial sequence		
<220>		

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 164th Cysteine is replaced with Alanine and the 299 Valine is replaced with Cysteine, and cDNA sequence coding thereof.

<400> 5

GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG	48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu	
1 5 10 15	
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG	96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys	
20 25 30	
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC	144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp	
35 40 45	
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG	192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln	
50 55 60	
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC	240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn	
65 70 75 80	
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC	288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly	
85 90 95	
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT	336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys	
100 105 110	
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA	384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr	
115 120 125	
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA	432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg	
130 135 140	
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC	480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro	
145 150 155 160	
AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG	528
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln	
165 170 175	
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCG	576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala	
180 185 190	
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG	624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu	
195 200 205	
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG	672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg	
210 215 220	
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CGG GGC ACC ACC AAC	720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn	
225 230 235 240	
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC	768

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
 245 250 255
 CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG 864
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
 275 280 285
 GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG TGC CTC AAC GTG CCC CGG 912
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg
 290 295 300
 CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
 305 310 315 320
 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
 325 330 335
 AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC 1056
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
 340 345 350
 CCG GGC ACC TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC 1104
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
 355 360 365
 GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC 1152
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
 370 375 380
 GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC 1200
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
 385 390 395 400
 CTG CGA GCC CCA TTT CCC TAG 1221
 Leu Arg Ala Pro Phe Pro
 405
 <210> 6
 <211> 406
 <212> PRT
 <213> artificial sequence
 <220>
 <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 164th Cysteine is replaced with Alanine and the 299 Valine is replaced with Cysteine.
 <400> 6
 Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
 1 5 10 15
 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
 20 25 30
 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
 35 40 45
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
 50 55 60
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn

65 70 75 80
 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
 85 90 95
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
 100 105 110
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
 115 120 125
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
 130 135 140
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
 145 150 155 160
 Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
 165 170 175
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
 180 185 190
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
 195 200 205
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
 210 215 220
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
 225 230 235 240
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
 245 250 255
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
 275 280 285
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg
 290 295 300
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
 305 310 315 320
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
 325 330 335
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
 340 345 350
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
 355 360 365
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
 370 375 380
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
 385 390 395 400
 Leu Arg Ala Pro Phe Pro
 405
 <210> 7
 <211> 1221
 <212> DNA
 <213> artificial sequence
 <220>
 <223> Amino acid sequence of recombinant mutant of blood coagulation fac

tor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu, and cDNA sequence coding thereof.

<400> 7

GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG	48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu	
1 5 10 15	
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG	96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys	
20 25 30	
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC	144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp	
35 40 45	
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG	192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln	
50 55 60	
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC	240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn	
65 70 75 80	
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC	288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly	
85 90 95	
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT	336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys	
100 105 110	
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA	384
Arg Cys Ile Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr	
115 120 125	
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA	432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg	
130 135 140	
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC	480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro	
145 150 155 160	
AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG GTG AAT GGA GCT CAG	528
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln	
165 170 175	
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC	576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala	
180 185 190	
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG	624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu	
195 200 205	
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG	672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg	
210 215 220	
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC	720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn	
225 230 235 240	
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC	768

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp			
245	250	255	
CAT GTG CTG CCC CTC TCG CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG			816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr			
260	265	270	
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG			864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu			
275	280	285	
GAC CGT GGC GCC ACG GCC CTC GAG CTC ATG GTG CTC AAC GTG CCC CGG			912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg			
290	295	300	
CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC			960
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser			
305	310	315	320
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC			1008
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser			
325	330	335	
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC			1056
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr			
340	345	350	
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC			1104
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys			
355	360	365	
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC			1152
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile			
370	375	380	
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC			1200
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu			
385	390	395	400
CTG CGA GCC CCA TTT CCC TAG			1221
Leu Arg Ala Pro Phe Pro			

405

<210> 8

<211> 406

<212> PRT

<213> artificial sequence

<220>

<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu.

<400> 8

Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu

1 5 10 15

Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys

20 25 30

Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp

35 40 45

Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln

50 55 60

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65 70 75 80
 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
 85 90 95
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
 100 105 110
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
 115 120 125
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
 130 135 140
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
 145 150 155 160
 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
 165 170 175
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
 180 185 190
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
 195 200 205
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
 210 215 220
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn
 225 230 235 240
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
 245 250 255
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
 275 280 285
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
 290 295 300
 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
 305 310 315 320
 Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
 325 330 335
 Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
 340 345 350
 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
 355 360 365
 Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
 370 375 380
 Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
 385 390 395 400
 Leu Arg Ala Pro Phe Pro
 405
 <210> 9
 <211> 1206
 <212> DNA
 <213> artificial sequence
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 <223> Amino acid sequence of recombinant mutant of blood coagulation fac

tor VII in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys, and cDNA sequence coding thereof.

<400> 9

GCC AAC GCG TTC CTG GAG GAG CTG CCG CCG GGC TCC CTG GAG AGG GAG	48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu	
1 5 10 15	
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CCG GAG ATC TTC AAG	96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys	
20 25 30	
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC	144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp	
35 40 45	
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG	192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln	
50 55 60	
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC	240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn	
65 70 75 80	
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC	288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly	
85 90 95	
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT	336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys	
100 105 110	
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA	384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr	
115 120 125	
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA	432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg	
130 135 140	
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC	480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro	
145 150 155 160	
AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG	528
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln	
165 170 175	
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC	576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala	
180 185 190	
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG	624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu	
195 200 205	
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG	672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg	
210 215 220	
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC	720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn	
225 230 235 240	
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC	768

His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
 245 250 255
 CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG 864
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
 275 280 285
 GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
 290 295 300
 CTG ATG ACC CAG GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG 960
 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
 305 310 315 320
 TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG 1008
 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
 325 330 335
 GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG TGG TAC 1056
 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
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 CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC 1104
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 355 360 365
 TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG 1152
 Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
 370 375 380
 CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TTT 1200
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 385 390 395 400
 CCC TAG 1206
 Pro
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 <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.
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 Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
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 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
 20 25 30
 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
 35 40 45
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
 50 55 60
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65 70 75 80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
 85 90 95
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
 100 105 110
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
 115 120 125
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
 130 135 140
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
 145 150 155 160
 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
 165 170 175
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
 180 185 190
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
 195 200 205
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
 210 215 220
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
 225 230 235 240
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
 245 250 255
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
 275 280 285
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
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 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
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 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
 325 330 335
 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
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 Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
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 <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Gl

u-Ala-Ser-Tyr-Pro-Gly-Lys, and cDNA sequence coding thereof.

<400> 11

GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG	48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu	
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TCC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG	96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys	
20 25 30	
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC	144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp	
35 40 45	
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG	192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln	
50 55 60	
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC	240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn	
65 70 75 80	
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC	288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly	
85 90 95	
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT	336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys	
100 105 110	
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA	384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr	
115 120 125	
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA	432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg	
130 135 140	
AAT GCC AGC AAA CCC CAA GCC CGA ATT GTG GGG GGC AAG GTG TGC CCC	480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro	
145 150 155 160	
AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG GTG AAT GGA GCT CAG	528
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln	
165 170 175	
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC	576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala	
180 185 190	
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG	624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu	
195 200 205	
GCC GAG CAC GAC CTC ACC GAG CAC GAC CGG GAT GAG CAG AGC CGG CGG	672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg	
210 215 220	
GTC GCG CAG GTC ATC ATC CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC	720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn	
225 230 235 240	
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC	768
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp	
245 250 255	

CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG 864
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
 275 280 285
 GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
 290 295 300
 CTG ATG ACC CAG GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG 960
 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
 305 310 315 320
 TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG 1008
 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
 325 330 335
 GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC CCG GGC ACG TGG TAC 1056
 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
 340 345 350
 CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC 1104
 Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
 355 360 365
 TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG 1152
 Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
 370 375 380
 CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TTT 1200
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 1 5 10 15
 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
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 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
 35 40 45
 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
 50 55 60
 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65 70 75 80

Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
 85 90 95
 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
 100 105 110
 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
 115 120 125
 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
 130 135 140
 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
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 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln
 165 170 175
 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
 180 185 190
 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
 195 200 205
 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
 210 215 220
 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn
 225 230 235 240
 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
 245 250 255
 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
 260 265 270
 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
 275 280 285
 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
 290 295 300
 Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
 305 310 315 320
 Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
 325 330 335
 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
 340 345 350
 Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
 355 360 365
 Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
 370 375 380
 Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe
 385 390 395 400
 Pro

【図面の簡単な説明】

【図1】 FVIIの一次構造及び改変部位（星印）を示す図。

【図2】 FVIIのプロテアーゼドメインアミノ酸配列を基にしたセリンプロテアーゼの基本構造を示す図。

【図3】 X線立体構造既知の各種トリプシン族セリンプロテアーゼ間の3Dマルチライメントを示す図。

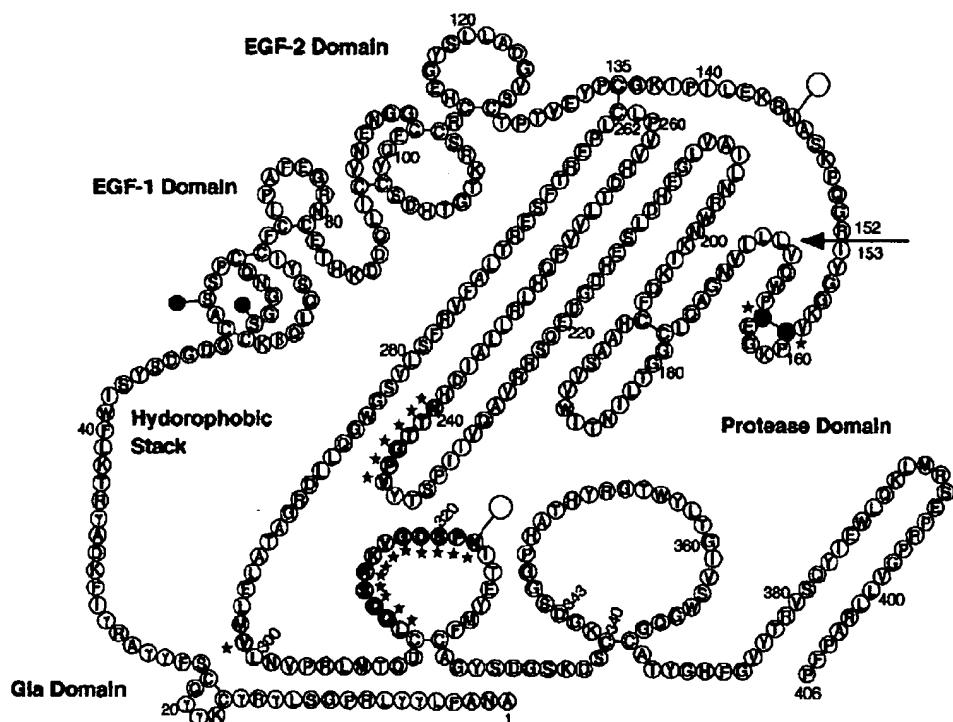
【図4】 野生型FVII (FVII-Wild) 及び各種FVII改変体のアミノ酸配列の一部を示す図。本

図はFVIIの153番目のイソロイシンよりC末側のアミノ酸配列のみ示したもので、152番目のアルギニンよりN末側のアミノ酸配列についてはいずれも改変は行っておらず野生型と同じである。

【図5】 FVII改変体作製用プライマー配列を示す図。

【図6】 FVII改変体発現ベクターの構築方法を示す図。

【図1】



【図2】

I V G G K V C P K G E C P W Q V L L I V N G A O L C G G T L I N T I W V V S A A 153	βストランド1	βストランド2	βストランド3 192
H C F D K I K N W R N L I A V L G E H D L S E H D G D E Q S R R V A Q V I P S 193	βストランド4	βストランド5	232
T Y V P G T T N H D I A L L R I H Q P V V L T D H V V P L C L P E R T F S E R T 233	βストランド6		272
L A F V R F S L V S G W G Q L L D R G A T A L E L M V L N V P R L M T Q D C L Q 273	βストランド7	βストランド8	312
Q S R K V G D S P N I T E Y M F C A G Y S D G S K D S C K G D S G G P H A T H Y 313	βストランド9	βストランド10	352
R G T W Y L T G I Y S W G Q G C A T Y G H F G V Y T R V S Q Y I E W L Q K L M R 353	βストランド11	βストランド12	392
S E P R P G V L L R A P F P 393			

【図3】

sequence 1: ヒト血液凝固第VII因子 (pdb ID 1DAN)
 sequence 2: ヒトトリプシン (pdb ID 1TRN)
 sequence 3: プタ血液凝固第IX因子 (pdb ID 1PFX)
 sequence 4: ウシトリプシン (pdb ID 1TLD)
 sequence 5: ヒト血液凝固第X因子 (pdb ID 1HCG)
 sequence 6: ヒトプロテインC (pdb ID 1AUT)
 sequence 7: プタカリクレインA (pdb ID NPKA)
 sequence 8: ウシキモトリプシン (pdb ID 5CHA)
 sequence 9: プタエラスター (pdb ID 3EST)
 sequence 10: ヒトαトロンビン (pdb ID 1PPB)
 sequence 11: ヒト多形核白血球プロテアーゼ3 (pdb ID 1FUJ)
 sequence 12: ラットトニン (pdb ID 1TON)
 sequence 13: ヒト好中球エラスター (pdb ID 1HNE)
 sequence 14: ヒトウロキナーゼ型プラスミノーゲンアクチベータ (pdb ID 1LMW)
 sequence 15: ヒトカテプシンG (pdb ID 1CGH)
 sequence 16: ラット肥満細胞プロテアーゼ (pdb ID 3RP2)
 sequence 17: ヒト組織型プラスミノーゲンアクチベータ (pdb ID 1RTF)

(図中@位置はすべてのプロテアーゼでのCα位置が1Å以内で保存されている構造保存部位を示す)

β ストランド5- β ストランド6近傍の アライメント	β ストランド8- β ストランド9近傍の アライメント
oooooooooooo:oooo:::oooo:oooooooooooo	oooooooooooo:oooo:::oooo:oooooooooooo
sequence 1: SRRVAQVIIIPSTYVP---G-TTNHDIALLRLHQ	ALELMVLNPRLMTQDCLQQSRKVGDSNPITEYMFAG
sequence 2: FINAAKIIRHPQYDR---K-TLNNDIMLKLSS	PDEOQLDAPVLSQAKCEA-S-Y---PGKITSNMFCAG
sequence 3: RRNVIIRAIPHHSYNAT---VNKYSHDIALLDELDE	ATILQYLKVLVDRATCL-R-ST-KFTIYSNMFCAG
sequence 4: F1SASKSIVHPHSYNS---N-TLNNDIMLKLKS	PDLKCLKAPI LSDSSCKS-A-Y---PGQITSNMFCAG
sequence 5: VHEVEVVIKHNRFTK---E-TYDFDIAVRLKT	STR1KMLEVYPVYDRNSCKL-S-S---SFIITQNMFCAG
sequence 6: DLDIKEVFVIIIPNYSK---S-TTDNDIALLHLAQ	TFVLNFIKIPVVPHNECSE-V-M---SNMVSENMLCAG
sequence 7: FFGVTADFPHPGFNLSA-DGKDYSHDLMLLRLQS	PDE1QCVQLTLLQNTFCA-D-AH-PDKVTESMLCAG
sequence 8: KLK1AKVKNSKYNS---L-TINNDITLLKLST	PDRLQQASLPLLSNTNCKK-Y-W-GTKIKDAMICAG
sequence 9: YVGVQKJVVHPYWNT--D-DVAAGYDIALLRLAQ	AQTLQQAYLPTVDYAJCSS-SSYW-GSTVKNSMVCAG
sequence 10: ISMLEKIIYIIIPRYNW---RENLDRDIALMKLKK	PSVLQVNLPIVERPVCKD-S-T---RIR1TDNMFCAG
sequence 11: HFSVAQVFLN-NYDA---E-NKLNDILLIQLSS	AOVLQELNVTWVT-FFC-----R-PHNICIF
sequence 12: RRLVRQSFHRPDVTP--LPVHDHSNDLMLLHLSE	SHDLQCVNTHILLSNEKCI-E-TY-KDNVTDVMLCAG
sequence 13: VFAVQRIFED-GYDP---V-NLLNDIVLQLNG	ASVLQELNVTWVT-SLC-----R-RSNVCTL
sequence 14: KFEVENLILHKDYSA--D-TLAHHNDIALLKIRS	PEQLRMTVVLISHRECQQPH-YY-GSEVTTKMLCAA
sequence 15: HITARRAIRHPQYNQ---R-T1QNDIMLQLLSR	TDTLREVQLRVQRDRQCLR-I-F---GSYDPRRQICVG
sequence 16: KIKVEKQIIHESYNS---V-PMLHDIMLKKLEK	SYTLREVELRIMDEKACVD-Y-R---Y-YEYKFQVCVG
sequence 17: KFEVEKYIVHKEFDD---D-TYDNDIALLQLKS	SERLKEAHVRLYPSSRCTSQH-LL-NRTVTDNMICAG

【図4】

VII-Wild

IVGGKCPKGECPWQVLLLVNGAQLCGGTINTIWWVSAAHCFDKIKNWRNLIAVLGEHD
 LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLTDHVVPLCLPERTFSERT
 LAFVRFLVSGWQQLLDRGATALELMVNVPRLMQDCLQQSRKVGDSPNITEYMFCAGY
 SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
 SEPRPGVLLRAPFP

VII-5

IVGGK~~V~~APKG~~E~~APWQVLLLVNGAQLCGGTINTIWWVSAAHCFDKIKNWRNLIAVLGEHD
 LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLTDHVVPLCLPERTFSERT
 LAFVRFLVSGWQQLLDRGATALELMVNVPRLMQDCLQQSRKVGDSPNITEYMFCAGY
 SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
 SEPRPGVLLRAPFP

VII-6

IVGGKCPKGECPWQVLLLVNGAQLCGGTINTIWWVSAAHCFDKIKNWRNLIAVLGEHD
 LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALRLHQPVVLTDHVVPLCLPERTFSERT
 LAFVRFLVSGWQQLLDRGATALELMCNVPRLMQDCLQQSRKVGDSPNITEYMFCAGY
 SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
 SEPRPGVLLRAPFP

VII-30

IVGGKCPKGECPWQVLLLVNGAQLCGGTINTIWWVSAAHCFDKIKNWRNLIAVLGEHD
 LSEHDGDEQSRRVAQVIIPSTYDRKTNHDIALRLHQPVVLTDHVVPLCLPERTFSERT
 LAFVRFLVSGWQQLLDRGATALELMVNVPRLMQDCLQQSRKVGDSPNITEYMFCAGY
 SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
 SEPRPGVLLRAPFP

VII-31

IVGGKCPKGECPWQVLLLVNGAQLCGGTINTIWWVSAAHCFDKIKNWRNLIAVLGEHD
 LSEHDGDEQSRRVAQVIIPSTYDRKTNHDIALRLHQPVVLTDHVVPLCLPERTFSERT
 LAFVRFLVSGWQQLLDRGATALELMVNVPRLMQDCEASYP-----GKITEYMFCAGY
 SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
 SEPRPGVLLRAPFP

VII-39

IVGGKCPKGECPWQVLLLVNGAQLCGGTINTIWWVSAAHCFDKIKNWRNLIAVLGEHD
 LSEHDGDEQSRRVAQVIIPSTYDRKTNHDIALRLHQPVVLTDHVVPLCLPERTFSERT
 LAFVRFLVSGWQQLLDRGATALELMVNVPRLMQDCEASYP-----GKITEYMFCAGY
 SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
 SEPRPGVLLRAPFP

(下線部は改変部位を表す)

【図5】

①VII-PWN Sense ; 5'-GGGGTCGACATGGCTCCAGGCCCTCAGGCTCCCTGCCTTCTG-3'

Factor VII Wild type のシグナル配列からのプライマーデザイン

5'-GGGGTCGACATGGCTCCAGGCCCTCAGGCTCCCTGCCTTCTG-3'

Sali M V S Q A L R L L C L L

②VII-PWC Antis ; 5'-CCCGGATCCCTAGGGAAATGGGCTCGCAGGAGGACTCCTGGCG-3'

Factor VII Wild type のカルボキシ末端までのプライマーデザイン

5'-CCCGGATCCCTAGGGAAATGGGCTCGCAGGAGGACTCCTGGCG-3'

BamHI

③VII-P5-1 Sense ; 5'-ATTGTGGGGCAAGGTGGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

④VII-P5-2 Antis ; 5'-GACCTGCCATGGGGCTCCCTTGGGGCACCTGCCAAT-3'

VII-5のプライマーデザイン (C159A, C164A)

5'-ATTGTGGGGCAAGGTGGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

3'-TAACACCCCGTCCACCGGGTTCCCTCCGGTACCGTCCAG-5'

I V G G K V A P K G E A P W Q V

⑤VII-P6-1 Sense ; 5'-TGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

⑥VII-P6-2 Antis ; 5'-GACCTGCCATGGGGCTCCCTTGGGC-3'

VII-6のプライマーデザイン① (C164A)

5'-TGCCCCAAAGGGGAGGCCCATGGCAGGTC-3'

3'-ACGGGTTTCCCCTCCGGGTACCGTCCAG-5'

C P K G E A P W Q V

⑦VII-P6-3 Sense ; 5'-CTGGAGCTCATGTGCCCTAACGTGCCCGG-3'

⑧VII-P6-4 Antis ; 5'-CCGGGCACGTTGAGGCACATGAGCTCCAG-3'

VII-6のプライマーデザイン② (V299C)

5'-CTGGAGCTCATGTGCCCTAACGTGCCCGG-3'

3'-GACCTCGAGTACACGGAGTTGACCGGGC-5'

L E L M C L N V P R

⑨VII-P30-1 Sense ; 5'-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3'

⑩VII-P30-2 Antis ; 5'-CAGGGCATGTCGGTTCAAGACTCTGCACCGACATCGCGCTG-3'

VII-30のプライマーデザイン (VPGTTN→DRKTLN)

5'-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3'

3'-TAGGGTCTGCATGTCCTCTGAGACTTGGTGTAGCGCAG-5'

I P S T Y D R K T L N H D I A L

⑪VII-P31-1 Sense ; 5'-ATGACCCAGGACTGCGAAGCCTCTACCCCTGGAAAAGATCACGGAGTACATG-3'

⑫VII-P31-2 Antis ; 5'-CATGTAATCCGTATCTTCCAGGGTAGGAGGCTCGCAGTCTGGCTCAT-3'

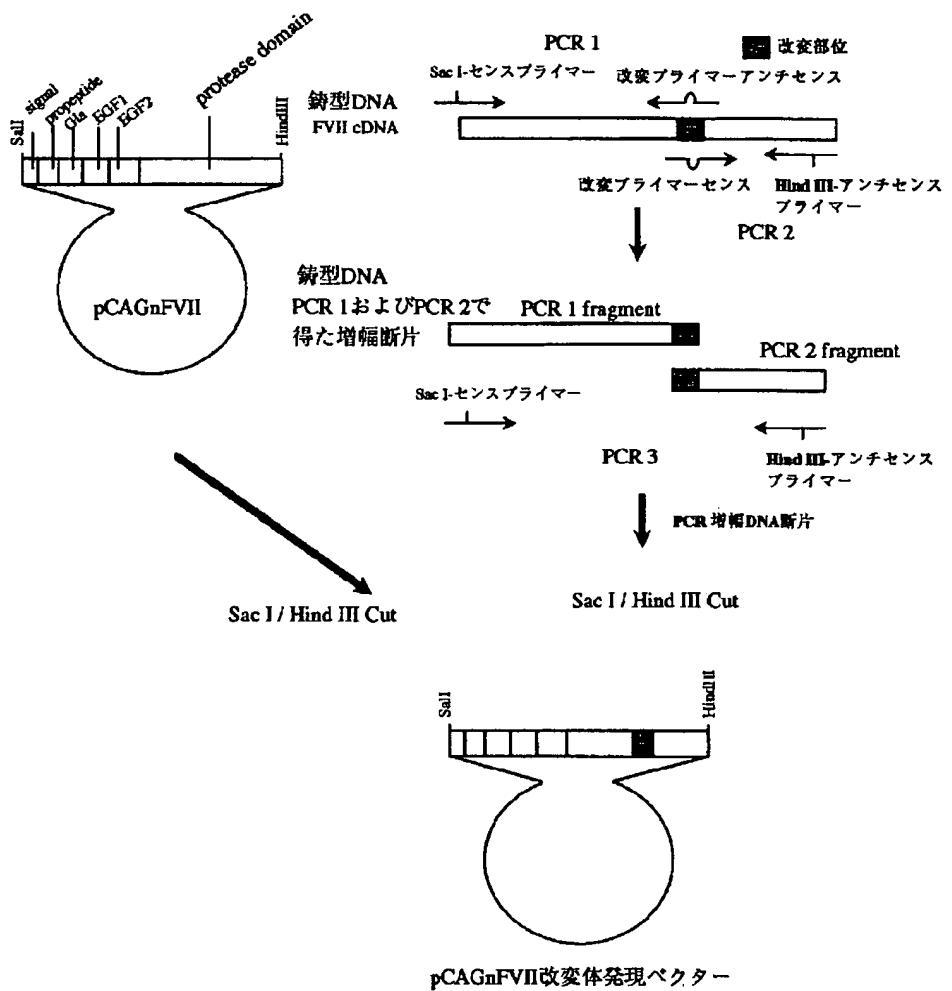
VII-31のプライマーデザイン (LQOSRKVGDSPN→EASYPGR)

5'-ATGACCCAGGACTGCGAAGCCTCTACCCCTGGAAAAGATCACGGAGTACATG-3'

3'-TACTGGGTCTGACGCTTCGGAGGATGGGACCTTCTAGTGCCTCATGTAC-5'

M T Q D C E A S Y P G K I T E Y M

【図6】



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EA04 GA11 GA13 GA18 HA01
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HH01 LL01
4C084 AA02 AA07 BA01 BA08 BA22
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